

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark
Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 25 February 1998 (25.02.98)	
International application No. PCT/IB97/01041	Applicant's or agent's file reference 50013/002WO1
International filing date (day/month/year) 03 July 1997 (03.07.97)	Priority date (day/month/year) 05 July 1996 (05.07.96)
Applicant BRANTON, Philip, E. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

05 February 1998 (05.02.98)

☐ in a notice effecting later election filed with the International Bureau on:
2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer G. Bähr Telephone No.: (41-22) 338.83.38
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From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT

(PCT Rule 71.1)

To:

McMAHON, Eileen M.
Deeth Williams Wall
National Bank Building
150 York Street, Suite 400
Toronto, Ontario M5H 3S5
CANADA

Date of mailing
(day/month/year)

01.12.98

Applicant's or agent's file reference
1994/0002

IMPORTANT NOTIFICATION

International application No.
PCT/IB97/01041

International filing date (day/month/year)
03/07/1997

Priority date (day/month/year)
05/07/1996

Applicant

BRANTON, Philip E. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/



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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)



Applicant's or agent's file reference 1994/0002	FOR FURTHER ACTION		See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
International application No. PCT/IB97/01041	International filing date (day/month/year) 03/07/1997	Priority date (day/month/year) 05/07/1996	
International Patent Classification (IPC) or national classification and IPC C12N15/34			
Applicant BRANTON, Philip E. et al.			

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 9 sheets, including this cover sheet.
 - ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☒ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 05/02/1998	Date of completion of this report 05.11.98
Name and mailing address of the IPEA/  European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer Stolz, B Telephone No. (+49-89) 2399-8416 

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB97/01041

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*:

Description, pages:

1-56 as originally filed

Claims, No.:

29-36, 46-53, 61-63, as originally filed
69, 70, 72-76, 78,
79

1-28, 37-45, 54-60, as received on
64-68

22/05/1998 with letter of 22/05/1998

Drawings, sheets:

1/19-19/19 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:
- ☐ copy of the earlier application whose priority has been claimed.

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/IB97/01041

- ☐ translation of the earlier application whose priority has been claimed.
2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.
- Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.
3. Additional observations, if necessary:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 3, 5-8, 13, 15-18, 23, 25-28, and partially 1, 2, 4, 9-12, 14, 19-22, 24, 29, 30.

because:

- ☒ the said international application, or the said claims Nos. relate to the following subject matter which do s not require an international preliminary examination (*specify*):

see separate sheet

- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☐ no international search report has been established for the said claims Nos. .

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:
- ☐ restricted the claims.

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- ☐ paid additional fees.
- ☐ paid additional fees under protest.
- ☐ neither restricted nor paid additional fees.
2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
- ☐ complied with.
- ☒ not complied with for the following reasons:
- see separate sheet**
4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:
- ☒ all parts.
- ☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1, 2, 4, 9-12, 14, 19-22, 24, 29-58, 68-70, 72-76, 78, 79
	No:	Claims	59-67
Inventive step (IS)	Yes:	Claims	11, 12, 14, 19-22, 24, 29-58, 69-70, 72-76, 78, 79
	No:	Claims	1, 2, 4, 9, 10, 59-67, 68
Industrial applicability (IA)	Yes:	Claims	1-79
	No:	Claims	

2. Citations and explanations

see separate sheet

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VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IB97/01041

1. Priority

- 1.1. Priority documents were not available at the time of establishing this preliminary opinion. This opinion has been established under the assumption of valid priority rights.

2. No opinion

- 2.1. Claims 3, 5-8, 13, 15-18, 23 and 25-28 in their entirety, and claims 1, 2, 4, 9-12, 24, 29 and 30 partially, refer to methods of therapeutic treatment which are carried out on the human or animal body. In agreement with Rule 67.1(iv) PCT the preliminary opinion is only established for the latter group of the above mentioned claims and only as far as in vitro methods are concerned.

3. Unity of Invention

- 3.1. The application appears to be lacking unity in the sense of Rules 13.1 and 13.2 PCT.

The present application discloses the role of two adenoviral proteins, E4orf6 and E4orf4, in inducing apoptosis in an p53 independent manner. Both proteins have been identified and sequenced previously (e.g. WO-A-96/14061, and Kleinberger and Shenk, 1993, cited in the ISR). Claimed are basically (i) methods of increasing apoptosis by expressing or introducing E4orf6 into cells, and pharmaceutical compositions comprising E4orf6 (claims 1-10, 21-44, 58, 69-71, 78 and 79), (ii) methods of increasing apoptosis by expressing or introducing E4orf4 into cells, and pharmaceutical compositions comprising E4orf4 (claims 11-30, 45-57, 72, 73, 78 and 79), (iii) methods for the identification of compounds (termed E4orf6 analogs) inducing apoptosis, and pharmaceutical compositions comprising such (claims 59, 61-67, 77, 80), (iv) methods for the identification of compounds (termed E4orf4 analogs) inducing apoptosis, and pharmaceutical compositions comprising such (claims 60-67, 77, 80), (v) a method to identify compounds acting on protein phosphatase 2a, and pharmaceutical compositions comprising such analogs (claims 68, 74-76). These five groups of inventions are not so linked as to form a single inventive concept. In particular, it is not apparent

wherein the required technical relationship among these inventions, which finds its expression in the claims in one or more of the same or corresponding special technical features (Rule 13.2 PCT), could be seen.

Nevertheless, in agreement with Rule 68.1 PCT, this preliminary opinion refers to all the claims.

4. Reasoned statement

4.1. Claims relating to the first group of inventions (group (i) as defined above):

Claims 1, 2, 4, 9 and 10 provide methods to induce apoptosis in cultured cells using the E4orf6 protein. The closest piece of prior art can be identified as Teodoro et al. (Oncogene 1995, cited in the ISR). It describes the induction of apoptosis in an p53 independent manner by the 289R E1A protein of adenoviruses. This protein transactivates one or several of the proteins encoded by the E2, E3 or E4 regions (p. 472 of Teodoro et al.) leading to apoptosis in cultured cells. The application differs from the prior art in that it specifies the gene or gene product, i.e. E4orf6, from the E4 region as inducing apoptosis. However, in light of WO-A-96/14061 (p. 13, lines 13-24), the selection of E4orf6 would have been obvious to the person skilled in the art trying to provide a method to induce apoptosis in cultured cells. Therefore claims 1, 2, 4, 9 and 10 are considered to lack inventive step.

On the other hand, claims 31 to 44, 58 and 69 to 71 appear to be novel and inventive, as the selective action of E4orf6 on cancerous cells and hence their use as pharmaceuticals was neither disclosed in nor made obvious from the available prior art.

4.2. Claims relating to the second group of inventions (group (ii) as defined above):

Claims 11, 12, 14, 19 to 22, 24, 29 and 30, and claims 45 to 57, 72, 73, 78 and 79 appear to be novel and inventive since the apoptosis inducing function as well as the selective mode of action of adenoviral E4orf4 was neither disclosed in nor made obvious from the available prior art.

4.3. Claims relating to the third and fourth groups of invention:

Claim 59 concerns a method of finding apoptosis inducing compounds, the method basically consisting of testing the compounds on cells expressing adenoviral E1A-289R protein. Thus any such compound, without necessarily involving the E4orf4 and/or E4orf6 pathway, is falling under these claims. According to the claims, any known apoptosis inducing substance having an effect on E1A-289R expressing cells should be termed an E4orf4 or E4orf6 analog. As they are presently worded, claims 59 to 67 are therefore considered to lack novelty.

4.4. Claims relating to the fifth group (group (v) as defined above):

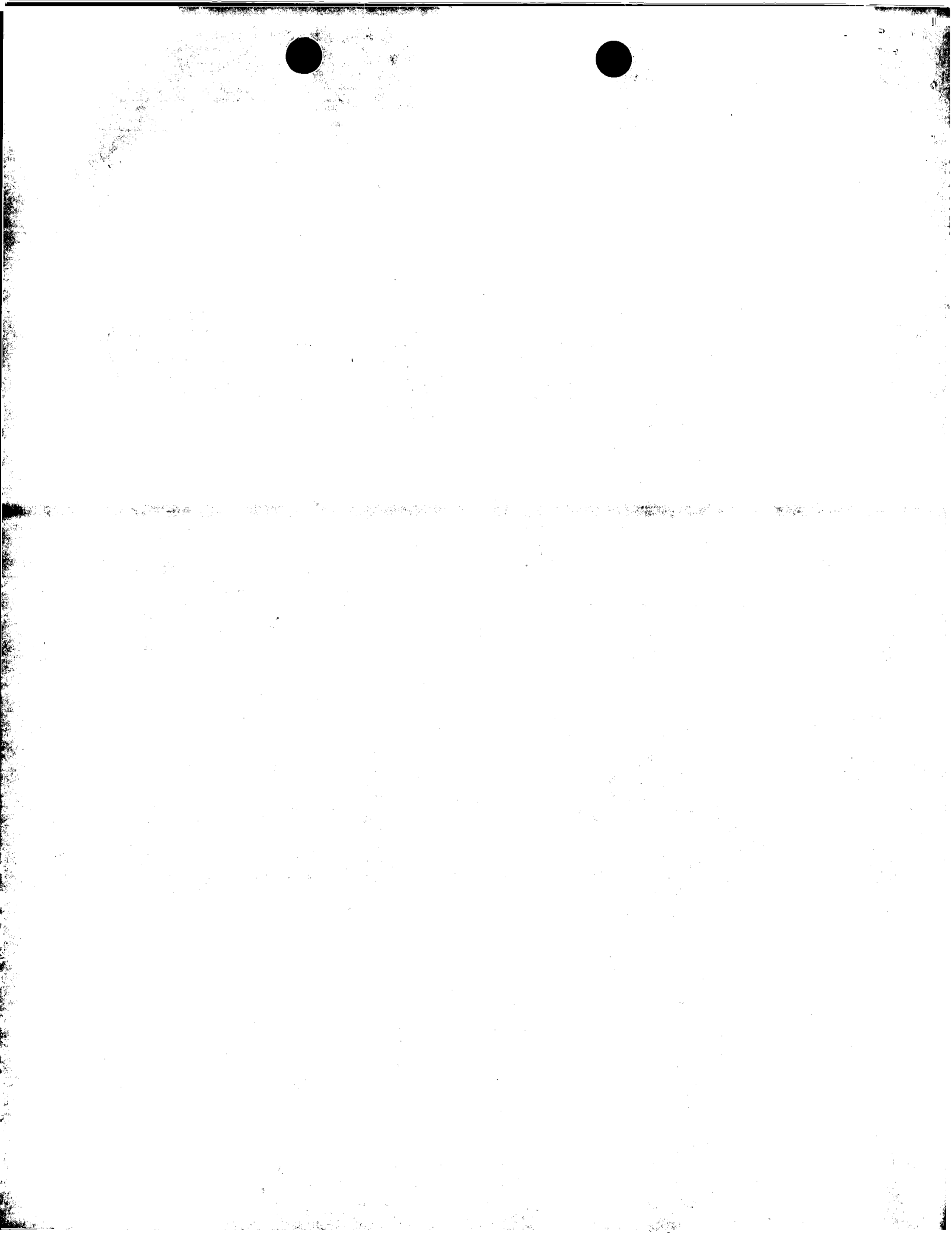
Claim 68 appears to be lacking inventive step (Art. 33(3) PCT). Claim 68 defines a procedure to identify compounds (termed E4orf4 analogs) that affect protein phosphatase 2a activity in cell lines expressing such phosphatase. The closest piece of prior art is Kleinberger and Shenk (1993, cited in the ISR) showing that E4orf4 binds to protein phosphatase 2a and affects transcription of junB. The paper provides assays to study activity of protein phosphatase 2a (see results). Claim 68 differs from the prior art in that the activity assays are used to identify additional compounds acting on protein phosphatase 2a. This however cannot be considered inventive, as it was the obvious way to find additional compounds acting on protein phosphatase 2a.

5. Certain documents

The following documents have been cited in the ISR (Rule 70.10 PCT): (a) WO-A-96/22378 filed on 19.01.1996, and claiming priority dates of 20.01.1995, 01.06.1995 and 08.09.1995, (b) WO-A-96/39530 filed on 04.06.1996, and claiming priority dates of 05.06.1995 and 27.10.1995.

6. Certain observations

- 6.1. The claims referring to E4orf4 and/or E4orf6 or such polypeptides do not comply with the requirements of Art. 6 PCT in combination with Rule 6.3 PCT. In the



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International application No. PCT/IB97/01041

absence of further specifications (e.g. sequence information, Seq. ID nos.), they do not contain the required technical features.

- 6.2. Claims 4, 14 and 24 do not appear to be fully supported by the description (Art. 6 PCT). The application does not provide any examples of compounds to be administered, apart from mRNA coding for E4orf4 and/or E4orf6 the above mentioned proteins.
- 6.3. Claims 74 to 76 do not appear to meet the requirements of Art. 6 PCT and Rule 6.3 PCT. The claims contain no technical features specifying the compounds to be used in the pharmaceutical compositions. The description does not provide any examples, apart from E4orf4 which is already claimed in claim 45. They define merely the result to be achieved. The claims appear thus not to be fully supported by the description.
- 6.4. Claims 2, 12 and 22 do not appear to specify features of the claimed procedure, which consists basically of administering an E4orf4 and/or E4orf6 to a cell, but specify rather the mode of action of these proteins. The meaning of these claims is thus unclear (Art. 6 PCT).

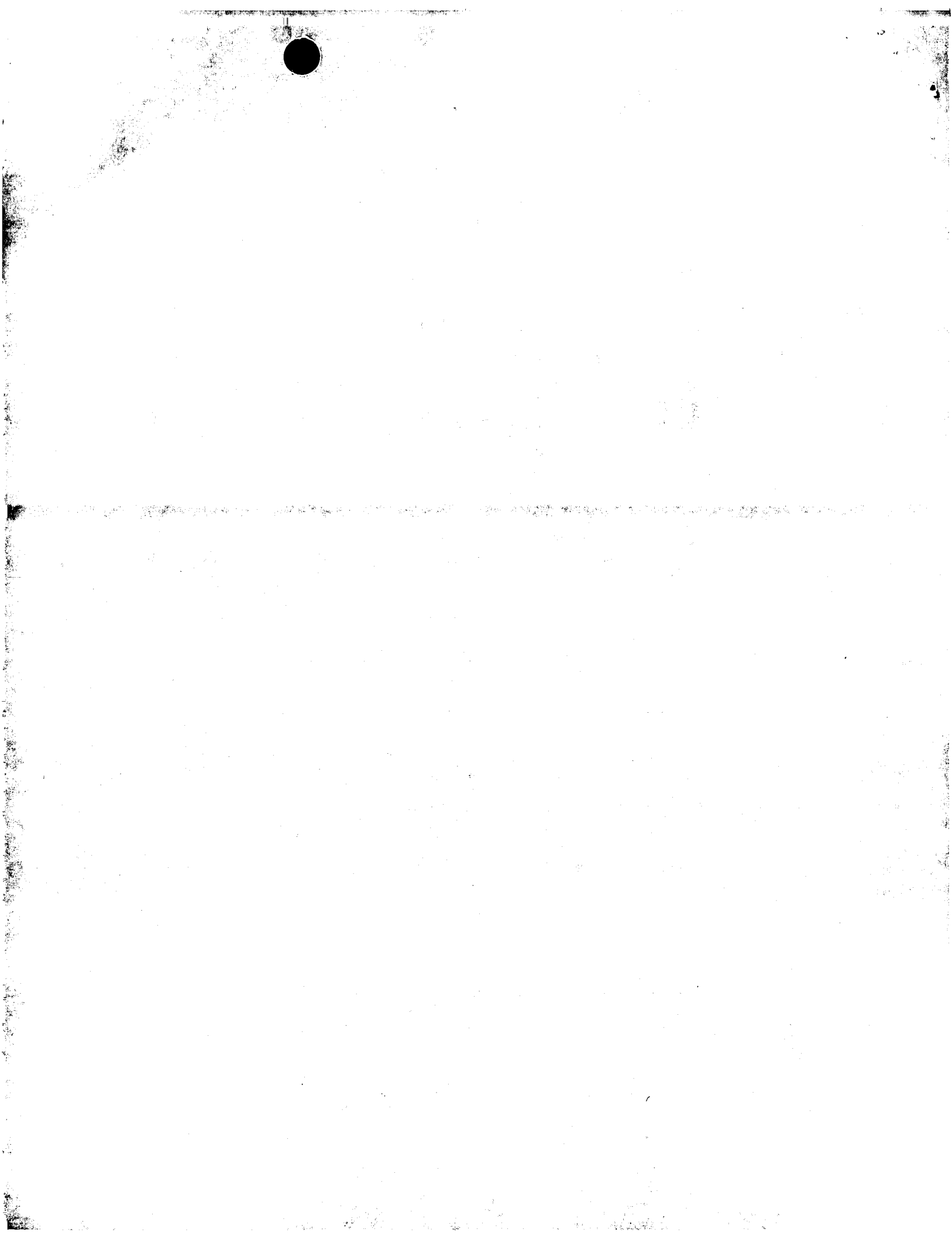
Several of the claims (e.g. 38, 42, 47 or 52) refer to "substantially identical" DNA or amino acid sequences. This term is vague and renders the scope of the claims unclear.

Claim 44 refers to fragments of E4orf6 without a functional limitation.

CLAIMS

1. A method of increasing apoptosis in a cell, said method comprising administering to said cell an apoptosis inducing amount of an E4orf6 polypeptide or
5 apoptotic fragment thereof.
2. The method of claim 1, wherein said apoptosis is p53-independent.
3. A method of increasing apoptosis in a mammal, said method comprising providing a transgene encoding an apoptotic E4orf6 polypeptide or an apoptotic fragment thereof to a cell of said mammal, said transgene being positioned
10 for expression in said cell.
4. A method of increasing apoptosis in a cell, said method comprising administering a compound which increases E4orf6 mediated E4orf6 biological activity.
5. The method of claim 1 or 4, wherein said cell is in a mammal.
- 15 6. The method of claim 5, wherein said mammal is a human.
7. The method of claim 1, 3, or 4, wherein said cell is in a mammal diagnosed as having a disease involving insufficient apoptosis.
8. The method of claim 7, wherein said disease is cancer.
9. The method of claim 4, wherein said compound is E4orf6 mRNA.
- 20 10. The method of claim 4, wherein said compound increases stability of E4orf6.

AMENDED SHEET



11. A method of increasing apoptosis in a cell, said method comprising administering to said cell an apoptosis inducing amount of an E4orf4 polypeptide or an apoptotic fragment thereof.

5 12. The method of claim 11, wherein said apoptosis is p53-independent.

13. A method of increasing apoptosis in a mammal, said method comprising providing a transgene encoding an apoptotic E4orf4 polypeptide or an apoptotic fragment thereof to a cell of said mammal, said transgene being positioned for expression in said cell.

10 14. A method of increasing apoptosis in a cell, said method comprising administering a compound which increases E4orf6 mediated E4orf6 biological activity.

15. The method of claim 11 or 14, wherein said cell is in a mammal.

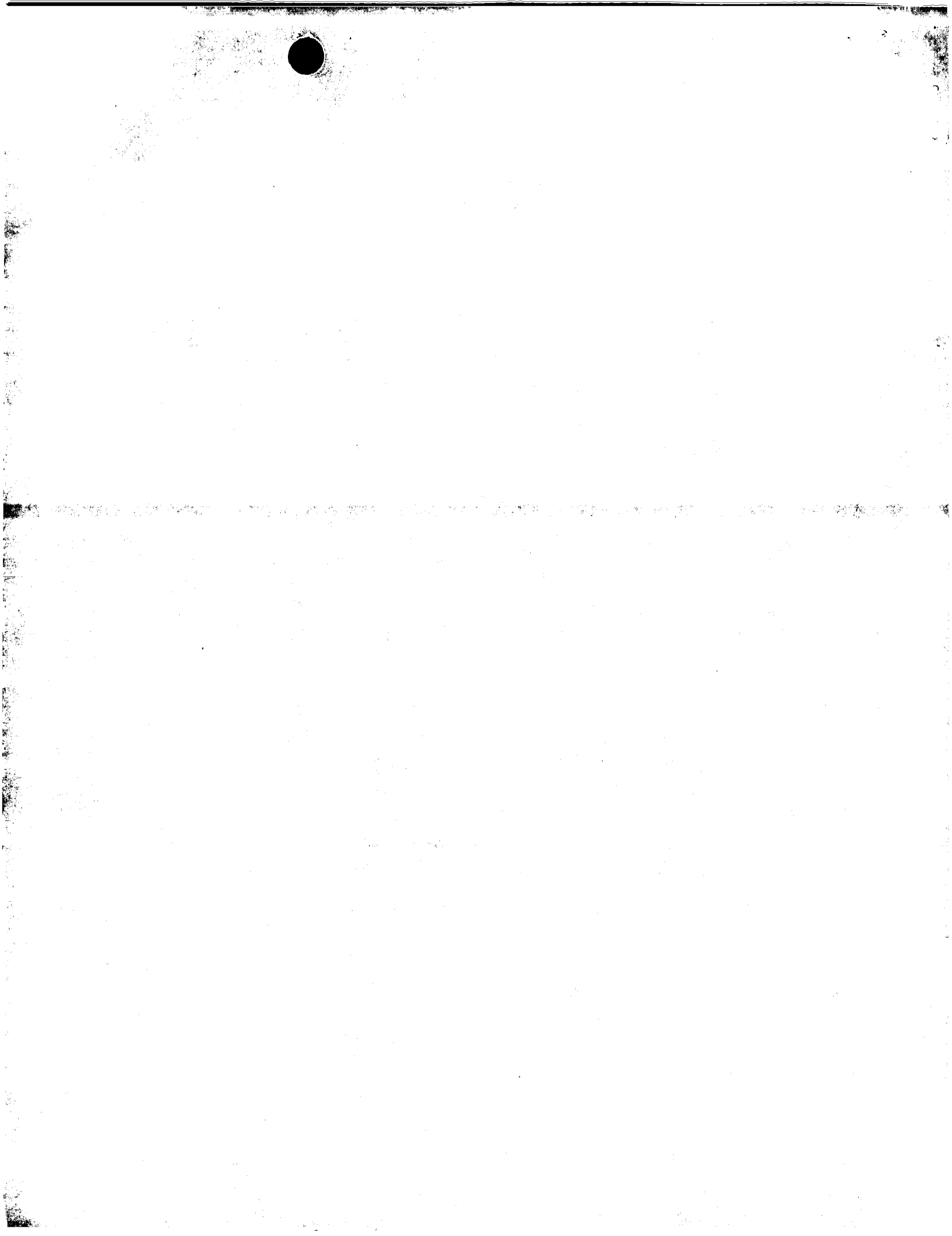
16. The method of claim 15, wherein said mammal is a human.

15 17. The method of claim 11, 13, or 14, wherein said cell is in a mammal diagnosed as having a disease involving insufficient apoptosis.

18. The method of claim 17, wherein said disease is cancer.

19. The method of claim 14, wherein said compound is E4orf4 mRNA.

20 20. The method of claim 14, wherein said compound increases the stability of E4orf4.



21. A method of increasing apoptosis in a cell, said method comprising administering to said cell an apoptosis inducing amount of a composition comprising and E4orf6 polypeptide or an apoptotic fragment thereof and an E4orf4 polypeptide or
5 an apoptotic fragment thereof.

22. The method of claim 21, wherein said apoptosis is p53-independent.

23. A method of increasing apoptosis in a mammal, said method comprising providing a first transgene encoding an E4orf6 polypeptide or an apoptotic fragment thereof and a second transgene encoding an E4orf4 polypeptide or an apoptotic
10 fragment thereof to a cell of said mammal, said first transgene and said second transgene being positioned for expression in said cell.

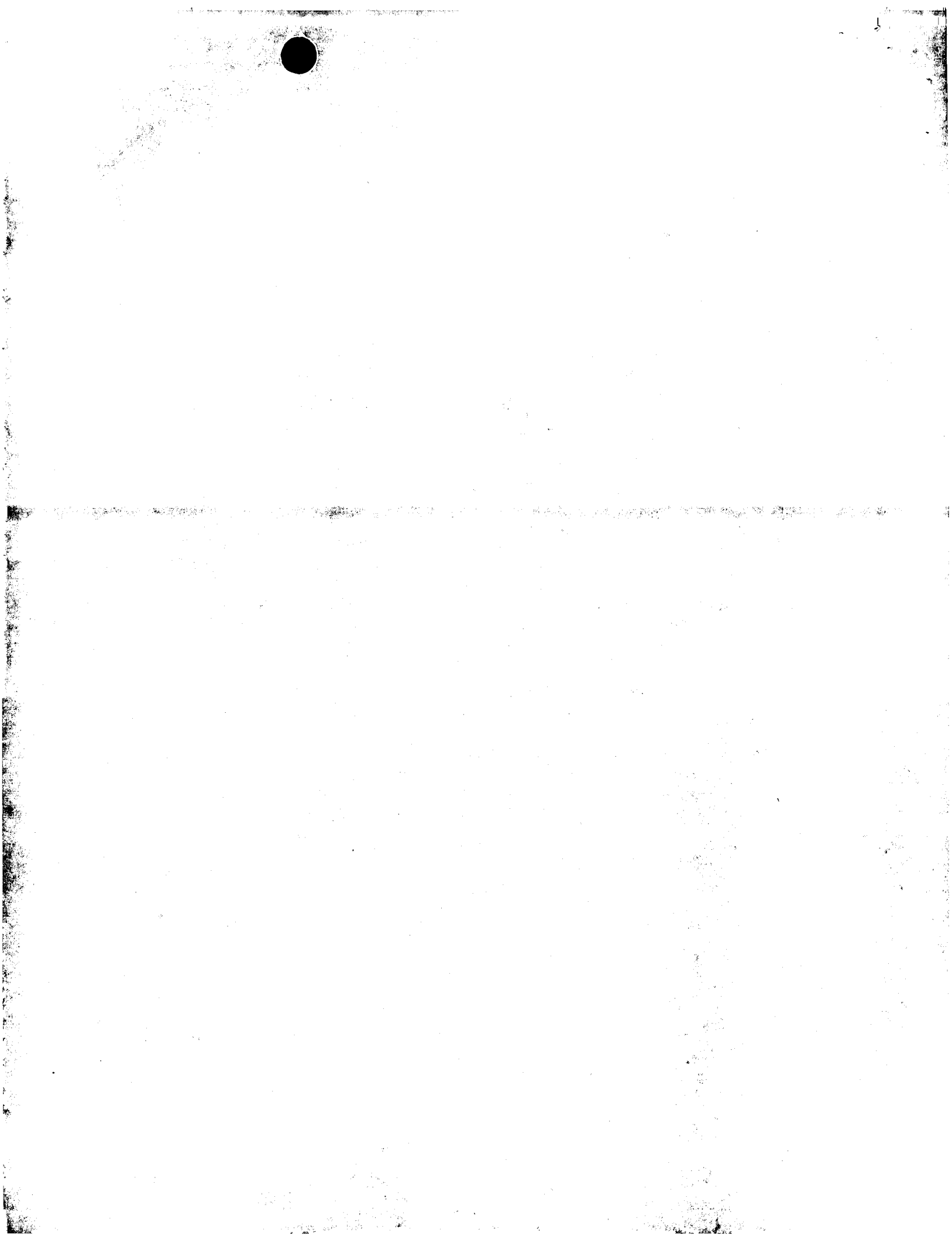
24. A method of increasing apoptosis in a cell, said method comprising administering a composition comprising a first compound which increases E4orf6 mediated E4orf6 biological activity and a second compound which increases E4orf6
15 mediated E4orf4 biological activity.

25. The method of claim 21 or 24, wherein said cell is in a mammal.

26. The method of claim 25, wherein said mammal is a human.

27. The method of claim 21, 23, or 24, wherein said cell is in a mammal diagnosed as having cancer.

20 28. The method of claim 23, wherein said first transgene and said second transgene encodes E4orf4 are linked to a constitutive promoter.



37. The composition of claim 36, wherein said nucleotide sequence identity is 75% or greater to the DNA sequence of Fig. 15 (SEQ ID NO.: 1).

5 38. A pharmaceutical composition comprising a DNA sequence substantially identical to the DNA sequence shown in Fig. 15 (SEQ ID NO.: 1).

39. The composition of claim 31 or 33, wherein said nucleic acid is operably linked to regulatory sequences for expression of said polypeptide and wherein said regulatory sequences comprise a promoter.

10 40. The composition of claim 39, wherein said promoter is a constitutive promoter, is inducible by one or more external agents, or is cell-type specific.

41. A pharmaceutical composition comprising substantially pure E4orf6
15 polypeptide, or an apoptotic fragment thereof.

42. The composition of claim 41, wherein said polypeptide comprises an amino acid sequence substantially identical to an amino acid sequence shown in Fig.15 (SEQ ID NO.: 2).

20 43. The composition of claim 41, wherein said polypeptide has a conservative amino acid substitution relative to the E4orf6 sequence of Fig. 15 (SEQ ID NO.: 2)

44. A pharmaceutical composition comprising a substantially pure polypeptide fragment of E4orf6.

25 45. A pharmaceutical composition comprising substantially pure nucleic acid encoding an E4orf4 polypeptide and a pharmaceutically acceptable carrier.

AMENDED SHEET

54. The composition of claim 53, wherein said promoter is a constitutive
5 promoter, is inducible by one or more external agents, or is cell-type specific.

55. A pharmaceutical composition comprising substantially pure mammalian
E4orf4 polypeptide, or an apoptotic fragment thereof

56. The composition of claim 55, said polypeptide comprising an amino acid
sequence substantially identical to an amino acid sequence shown in Fig. 16 (SEQ ID
10 NO.: 4).

57. The composition of claim 55, wherein said polypeptide has a conservative
amino acid substitution relative to the E4orf4 sequence of Fig. 16 (SEQ ID NO.: 4).

58. A pharmaceutical composition comprising a substantially pure polypeptide
fragment of E4orf4.

15 59. A method for identifying a compound as an E4orf6 or E4orf4 analog, said
method comprising the steps of:

- a) providing a cell expressing the adenovirus E1A-289R protein, said cell not
expressing any E4 proteins;
- b) contacting said cell with a candidate compound; and
- 20 c) determining viability of said cell, wherein apoptotic death of said cell
indicates a compound that is an E4orf6 analog.

60. A method for identifying a compound as an E4orf4 analog, said method
comprising the steps of:

- a) providing a cell expressing the adenovirus E1A-289R protein, said cell not
25 expressing any E4 proteins;

b) contacting said cell with a candidate compound; and
c) determining viability of said cell, wherein apoptotic death of said cell indicates a compound that is an E4orf4 analog.

5 61. The method of claim 59, wherein said cell is selected from the group consisting of: 1A.A3, 1A.A6, and 1A.A12 cells.

62. The method of claim 59, wherein said viability is measured with Trypan Blue™.

10 63. The method of claim 59, wherein said viability is measured with a DNA fragmentation assay.

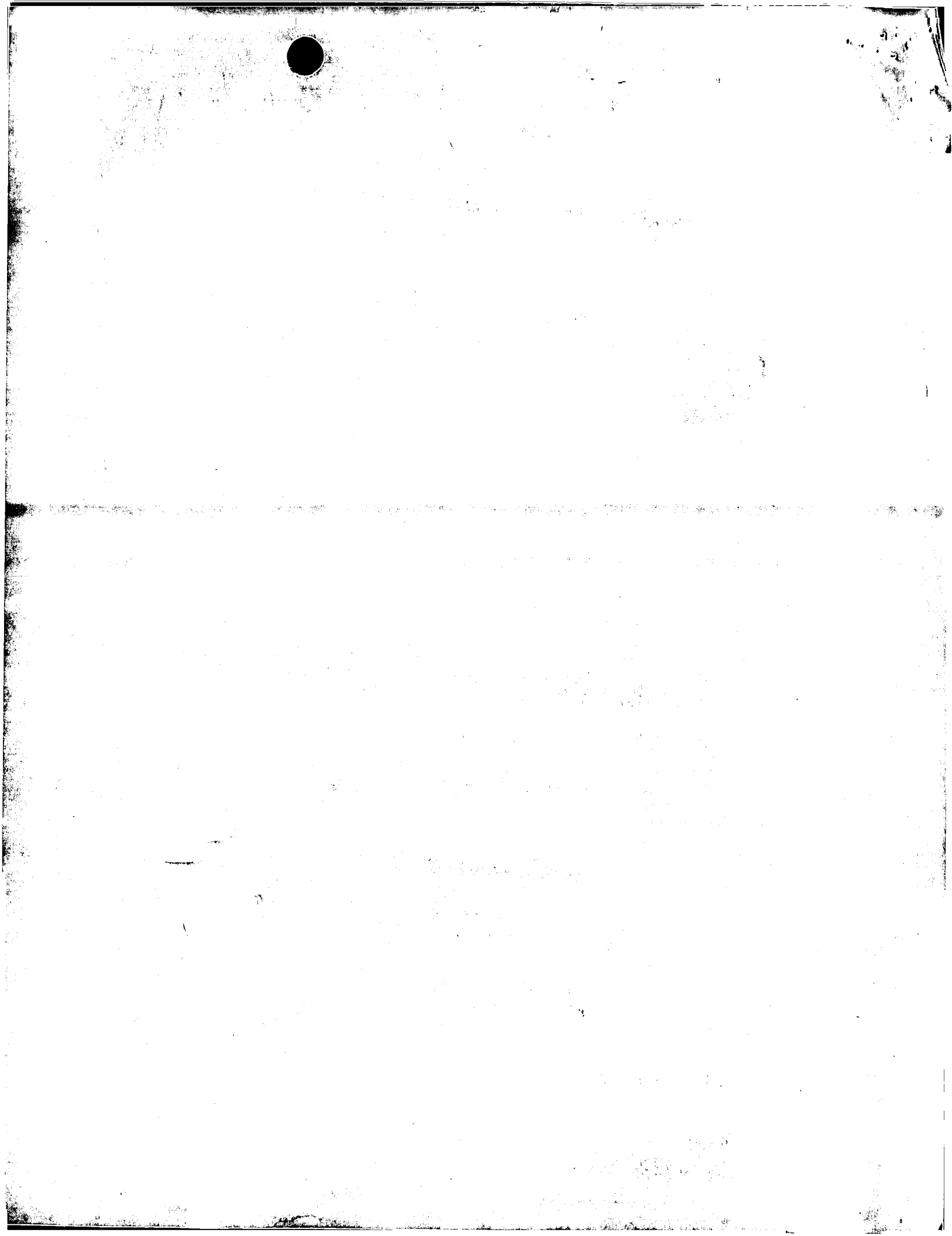
64. The method of claim 59, wherein said viability is measured with Annexin V binding.

65. The method of claim 59, wherein said viability is measured with Propidium Iodide

15 66. The method of claim 59, wherein said viability is measured utilizing a combination of two or more of the following: Trypan Blue™, DNA fragmentation, Annexin V binding, and Propidium Iodide.

67. The method of claim 59, wherein said cell is a cell infected with a mutant adenovirus, said mutant adenovirus incapable of expressing any E4 proteins.

20 68. A method for identifying a compound as an E4orf4 analog, said method comprising the steps of:



CLAIMS

1. A method of increasing apoptosis in a cell, said method comprising administering to said cell an apoptosis inducing amount of an E4orf6 polypeptide or apoptotic fragment thereof.

5 2. The method of claim 1, wherein said apoptosis is p53-independent.

3. A method of increasing apoptosis in a mammal, said method comprising providing a transgene encoding an apoptotic E4orf6 polypeptide or an apoptotic fragment thereof to a cell of said mammal, said transgene being positioned for expression in said cell.

10 4. A method of increasing apoptosis in a cell, said method comprising administering a compound which increases E4orf6 biological activity.

5. The method of claim 1 or 4, wherein said cell is in a mammal.

6. The method of claim 5, wherein said mammal is a human.

15 7. The method of claim 1, 3, or 4, wherein said cell is in a mammal diagnosed as having a disease involving insufficient apoptosis.

8. The method of claim 7, wherein said disease is cancer.

9. The method of claim 4, wherein said compound is E4orf6 mRNA.

10. The method of claim 4, wherein said compound increases stability of E4orf6.

11. A method of increasing apoptosis in a cell, said method comprising administering to said cell an apoptosis inducing amount of an E4orf4 polypeptide or an apoptotic fragment thereof.

12. The method of claim 11, wherein said apoptosis is p53-independent.

5 13. A method of increasing apoptosis in a mammal, said method comprising providing a transgene encoding an apoptotic E4orf4 polypeptide or an apoptotic fragment thereof to a cell of said mammal, said transgene being positioned for expression in said cell.

10 14. A method of increasing apoptosis in a cell, said method comprising administering a compound which increases E4orf4 biological activity.

15. The method of claim 11 or 14, wherein said cell is in a mammal.

16. The method of claim 15, wherein said mammal is a human.

17. The method of claim 11, 13, or 14, wherein said cell is in a mammal diagnosed as having a disease involving insufficient apoptosis.

15 18. The method of claim 17, wherein said disease is cancer.

19. The method of claim 14, wherein said compound is E4orf4 mRNA.

20. The method of claim 14, wherein said compound increases the stability of E4orf4.

21. A method of increasing apoptosis in a cell, said method comprising administering to said cell an apoptosis inducing amount of a composition comprising an E4orf6 polypeptide or an apoptotic fragment thereof and an E4orf4 polypeptide or an apoptotic fragment thereof .

5 22. The method of claim 21, wherein said apoptosis is p53-independent.

23. A method of increasing apoptosis in a mammal, said method comprising providing a first transgene encoding an E4orf6 polypeptide or an apoptotic fragment thereof and a second transgene encoding an E4orf4 polypeptide or an apoptotic fragment thereof to a cell of said mammal, said first transgene and said second
10 transgene being positioned for expression in said cell.

24. A method of increasing apoptosis in a cell, said method comprising administering a composition comprising a first compound which increases E4orf6 biological activity and a second compound which increases E4orf4 biological activity.

25. The method of claim 21 or 24, wherein said cell is in a mammal.

15 26. The method of claim 25, wherein said mammal is a human.

27. The method of claim 21, 23, or 24, wherein said cell is in a mammal diagnosed as having cancer.

28. The method of claim 23, wherein said first transgene and said second transgene encodes E4orf4 are linked to a constitutive promoter.

37. The composition of claim 36, wherein said nucleotide sequence identity is 75% or greater to the DNA sequence of Fig. 15 (SEQ ID NO.: 1).

38. A pharmaceutical composition comprising a DNA sequence substantially identical to the DNA sequence shown in Fig. 15 (SEQ ID NO.: 1).

5 39. The composition of claim 31 or 33, wherein said nucleic acid is operably linked to regulatory sequences for expression of said polypeptide and wherein said regulatory sequences comprise a promoter.

40. The composition of claim 39, wherein said promoter is a constitutive promoter, is inducible by one or more external agents, or is cell-type specific.

10 41. A pharmaceutical composition comprising substantially pure mammalian E4orf6 polypeptide, or an apoptotic fragment thereof.

42. The composition of claim 41, wherein said polypeptide comprises an amino acid sequence substantially identical to an amino acid sequence shown in Fig. 15 (SEQ ID NO.: 2).

15 43. The composition of claim 41, wherein said polypeptide has a conservative amino acid substitution relative to the E4orf6 sequence of Fig. 15 (SEQ ID NO.: 2).

44. A pharmaceutical composition comprising a substantially pure polypeptide fragment of E4orf6.

20 45. A pharmaceutical composition comprising substantially pure nucleic acid encoding an E4orf4 polypeptide and a pharmaceutically acceptable carrier.

54. The composition of claim 53, wherein said promoter is a constitutive promoter, is inducible by one or more external agents, or is cell-type specific.

55. A pharmaceutical composition comprising substantially pure mammalian E4orf4 polypeptide, or an apoptotic fragment thereof.

5 56. The composition of claim 55, said polypeptide comprising an amino acid sequence substantially identical to an amino acid sequence shown in Fig. 16 (SEQ ID NO.: 4).

57. The composition of claim 55, wherein said polypeptide has a conservative amino acid substitution relative to the E4orf4 sequence of Fig. 16 (SEQ ID NO.: 4).

10 58. A pharmaceutical composition comprising a substantially pure polypeptide fragment of E4orf6.

59. A method for identifying a compound as an E4orf6 analog, said method comprising the steps of:

15 a) providing a cell expressing the adenovirus E1A-289R protein, said cell not expressing any E4 proteins;

b) contacting said cell with a candidate compound; and

c) determining viability of said cell, wherein apoptotic death of said cell indicates a compound that is an E4orf6 analog.

20 60. A method for identifying a compound as an E4orf4 analog, said method comprising the steps of:

a) providing a cell expressing the adenovirus E1A-289R protein, said cell not expressing any E4 proteins;

b) contacting said cell with a candidate compound; and
c) determining viability of said cell, wherein apoptotic death of said cell indicates a compound that is an E4orf4 analog.

61. The method of claim 59 or 60, wherein said cell is selected from the group consisting of: 1A.A3, 1A.A6, and 1A.A12 cells.

62. The method of claim 59 or 60, wherein said viability is measured with Trypan Blue™.

63. The method of claim 59 or 60, wherein said viability is measured with a DNA fragmentation assay.

64. The method of claim 59 or 60, wherein said viability is measured with Annexin V binding.

65. The method of claim 59 or 60, wherein said viability is measured with Propidium Iodide

66. The method of claim 59 or 60, wherein said viability is measured utilizing a combination of two or more of the following: Trypan Blue™, DNA fragmentation, Annexin V binding, and Propidium Iodide.

67. The method of claim 59 or 60, wherein said cell is a cell is infected with a mutant adenovirus, said mutant adenovirus incapable of expressing any E4 proteins.

68. A method for identifying a compound as an E4orf4 analog, said method comprising the steps of:

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 1994/0002	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/IB 97/01041	International filing date (day/month/year) 03/07/1997	(Earliest) Priority Date (day/month/year) 05/07/1996

Applicant

BRANTON, Philip E. et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. ☒ Certain claims were found unsearchable (see Box I).

2. ☐ Unity of invention is lacking (see Box II).

3. ☒ The international application contains disclosure of a nucleotide and/or amino acid sequence listing and the international search was carried out on the basis of the sequence listing

☒ filed with the international application.

☐ furnished by the applicant separately from the international application,

☐ but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

☐ Transcribed by this Authority

4. With regard to the title, ☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

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☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is:

Figure No. _____ ☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 97/01041

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 3 5-8 13 15-18 23 25-28 and 1 2 4 9-12 14 19-22 24 29 30 ,as far as they concern an in vivo method , are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/97/01041

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/34 C12N15/86 C07K14/075 G01N33/68 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 14061 A (CELL GENESYS INC) 17 May 1996	31-54
Y	see page 13, line 13 - line 19; claims 1-59	1,4,9, 11,14, 21,24, 29, 55-58, 68-80
X	--- WO 96 13596 A (RHONE POULENC RORER SA ;YEH PATRICE (FR); PERRICAUDET MICHEL (FR);) see claims 1-37	31-54
X	--- BRIDGE E ET AL: "Adenovirus early region 4 and viral DNA synthesis." VIROLOGY, APR 1993, 193 (2) P794-801, UNITED STATES, XP002051623 see the whole document ---	31-54
	-/-	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

9 January 1998

Date of mailing of the international search report

03.02.98

Name and mailing address of the ISA

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03 05 38

INTERNATIONAL SEARCH REPORT

International Application No

PCT/97/01041

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KETNER G ET AL: "Complementation of adenovirus E4 mutants by transient expression of E4 cDNA and deletion plasmids." NUCLEIC ACIDS RES, APR 25 1989, 17 (8) P3037-48, ENGLAND, XP002051624 see the whole document	31-54
Y	TEODORO JG ET AL: "Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53-independent mechanisms." ONCOGENE, AUG 3 1995, 11 (3) P467-74, ENGLAND, XP002051625 see page 472, left-hand column, paragraph 2 - line END	1,4,9, 11,14, 21,24, 29, 69-73, 77-80
Y	HERISSE J ET AL: "Nucleotide sequence of adenovirus 2 DNA fragment encoding for the carboxylic region of the fiber protein and the entire E4 region." NUCLEIC ACIDS RES, AUG 25 1981, 9 (16) P4023-42, ENGLAND, XP002051626 see figure 2	55-58
Y	KLEINBERGER T ET AL: "Adenovirus E4orf4 protein binds to protein phosphatase 2A, and the complex down regulates E1A-enhanced junB transcription." J VIROL, DEC 1993, 67 (12) P7556-60, UNITED STATES, XP002051627 cited in the application see the whole document	68,73-76
P,X	MARCELLUS, RICHARD C. ET AL: "Adenovirus type 5 early region 4 is responsible for E1A-induced p53-independent apoptosis" J. VIROL. (1996), 70(9), 6207-6215 CODEN: JOVIAM;ISSN: 0022-538X, XP002051628 see the whole document	1,2,11, 12,14, 21,22, 24,31-54
P,X	WO 96 22378 A (RHONE POULENC RORER SA ;DEDIEU JEAN FRANCOIS (FR); LATTA MARTINE () see abstract; claims 1-50	31-34, 39,40
P,X	WO 96 39530 A (UNIV PENNSYLVANIA ;WILSON JAMES M (US); FISHER KRISHNA J (US); GAO see abstract; claims 1-23	31-34, 39,40

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/97/01041

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9614061 A	17-05-96	AU 4109296 A CA 2204357 A EP 0797436 A	31-05-96 17-05-96 01-10-97
WO 9613596 A	09-05-96	FR 2726285 A AU 3847795 A EP 0787199 A FI 971783 A NO 971764 A ZA 9509086 A	03-05-96 23-05-96 06-08-97 25-04-97 17-04-97 16-07-96
WO 9622378 A	25-07-96	FR 2729674 A FR 2741891 A FR 2738575 A AU 4544396 A CA 2210168 A EP 0805868 A FI 973055 A NO 973332 A	26-07-96 06-06-97 14-03-97 07-08-96 25-07-96 12-11-97 18-07-97 18-07-97
WO 9639530 A	12-12-96	AU 6277996 A	24-12-96



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/IB97/01041		(74) Agent: McMAHON, Eileen, M.; Deeth Williams Wall, National Bank Building, Suite 400, 150 York Street, Toronto, Ontario M5H 3S5 (CA).	
(22) International Filing Date: 3 July 1997 (03.07.97)		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
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(60) Parent Applications or Grants (63) Related by Continuation US 60/021,273 (CON) Filed on 5 July 1996 (05.07.96) US 60/028,740 (CON) Filed on 22 October 1996 (22.10.96)			
(71)(72) Applicants and Inventors: BRANTON, Philip, E. [CA/CA]; 266 Macaulay, St. Lambert, Quebec J4R 2G9 (CA). SHORE, Gordon, C. [CA/CA]; 4842 Wilson, Montreal, Quebec H3X 3P2 (CA). TEODORO, Jose, G. [CA/CA]; 3484 Stanley #610, Montreal, Quebec H3A 1S1 (CA). MARCELLUS, Richard, C. [CA/CA]; 3504 Durocher #2, Montreal, Quebec H2X 2E5 (CA). LAVOIE, Josee, N. [CA/CA]; 1100 Dr. Penfield #1416, Montreal, Quebec H3A 1A8 (CA).			
(54) Title: ADENOVIRUS E4 PROTEINS FOR INDUCING CELL DEATH			
(57) Abstract <p>The present invention relates to a pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which includes adenovirus E4orf4, an analog or a biologically active fragment thereof and/or adenovirus E4orf6, an analog or a biologically active fragment thereof. There is also provided a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of adenovirus E4orf4, an analog or a biologically active fragment thereof and/or adenovirus E4orf6, an analog or a biologically active fragment thereof in association with a pharmaceutical carrier. Included in the invention are methods for identifying compounds as analogs of the adenovirus E4 death proteins.</p>			

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ADENOVIRUS E4 PROTEINS FOR INDUCING CELL DEATH

BACKGROUND OF THE INVENTION

5

(a) Field of the Invention

The invention relates to a pharmaceutical agent(s) to induce cell death for use in treating conditions which involve inappropriate cell survival.

(b) Description of Prior Art

10

Replication of human adenoviruses in terminally differentiated epithelial cells requires an efficient mechanism to induce cellular DNA synthesis. This induction permits replication of viral DNA and production of progeny virus. Human adenoviruses infect and kill epithelial cells very efficiently. Cell death occurs by apoptosis and virus spread occurs through endocytosis by surrounding cells.

15

Products of early region 1A (E1A) of the adenovirus genome induce cell DNA synthesis and are largely responsible for cell transformation by adenoviruses. E1A produces two major mRNAs of 13S and 12S which encode proteins of 289 and 243 residues (289R and 243R, respectively) that are identical except for the lack in 243R of a central 46-amino acid sequence, termed conserved region 3 or CR3, as

20

schematically depicted in Fig. 1A. Two additional regions present in the common sequence encoded by exon 1 of both E1A mRNAs are also conserved in all human adenovirus serotypes and have been termed CR1 and CR2. E1A products induce DNA synthesis through complex formation between CR2 and CR1 and the

25

retinoblastoma tumor suppressor pRB and related p107 and p130 proteins, or between the amino terminus and CR1 and the transcriptional modulator p300 and possibly related proteins (Corbeil, H.B. et al., 1994, J. Virol. 68: 6697-6709). E1A-289R also activates expression of the early viral transcription units E2, E3, and E4, and certain cellular genes at least in part through interactions with transcription factors and basal

transcription machinery requiring CR3 (Teodoro, J.G. et al., 1995, *Oncogene* 11: 467-474). In addition to CR3, transactivation of the E4 promoter has also been shown to rely to some degree on two regions encoded by the second exon of 13S mRNA, termed auxiliary regions 1 and 2, or AR1 and AR2. Production of stably transformed
5 cells requires early region 1B (E1B) which encodes polypeptides of 19 and 55kDa that are individually capable of cooperating with E1A via separate but additive pathways (McLorie, W. et al., 1991, *J. Gen. Virol.* 72: 1467-1471).

Considerable evidence indicates that a major function of E1B proteins in lytic infection and cell transformation is to suppress cytotoxic effects and apoptosis induced
10 by expression of E1A. Without E1B, the toxicity of E1A products results in the death of E1A-transformed cells and a reduction in the yield of progeny due to the early demise of productively infected cells. E1A proteins can cause apoptosis by a process mediated by the tumor suppressor p53, which controls growth arrest and programmed cell death pathways (Teodoro, J.G. et al., 1995, *Oncogene* 11: 467-474). Expression
15 of E1A products results in the elevation of p53 levels. The 55kDa E1B protein binds to p53 and blocks both p53-mediated activation of gene expression and apoptosis (Teodoro, J.G. et al., 1994, *J. Virol.* 68: 776-786). The 19kDa E1B protein appears to suppress apoptosis by a mechanism that is functionally analogous to that of the cellular proto-oncogene product Bcl-2 (Nguyen, M. et al., 1994, *J. Biol. Chem.* 269: 16521-16524). Cells infected with adenovirus mutants which fail to express the
20 19kDa protein display enhanced cytotoxicity and extensive degradation of both cellular and viral DNA into nucleosome sized fragments (McLorie, W. et al., *supra*; Teodoro, J.G. et al., 1995, *Oncogene* 11: 467-474). At later times, even in the presence of E1B proteins, infected cells suffer apoptotic death and viral progeny
25 spread to neighboring cells through endocytosis of cell fragments. In addition to the induction of DNA synthesis and cell transformation, the large 289-residue (289R) E1A protein also transactivates expression of all early viral genes, including early regions 1A, 1B, 2, 3 and 4 (reviewed in Teodoro, J.G. et al., 1995, *Oncogene* 11:

467-474).

It would be highly desirable to be provided with a pharmaceutical agent for induction of apoptosis when such induction is useful in the treatment of human diseases which involve inappropriate cell survival.

5

SUMMARY OF THE INVENTION

In accordance with the present invention, we have used a genetic approach to identify the role of individual E4 proteins in the induction of p53-independent apoptosis. Our results indicate the E4 death proteins, E4orf4 or E4orf6, are responsible for induction of p53-apoptosis in transformed, but not untransformed, cells. Thus, E4orf4 and E4orf6 are both powerful inducers of p53-independent cell death. This discovery has significant ramifications for both apoptosis-inducing therapeutics and drug screens.

In a first aspect, the invention provides a method of increasing apoptosis in a cell by administering to the cell an apoptosis inducing amount of an E4orf6 polypeptide or an apoptotic fragment thereof. In a preferred embodiment of this aspect, the apoptosis is p53-independent.

In a second aspect, the invention provides a method of increasing apoptosis in a mammal which includes providing a transgene encoding an E4orf6 polypeptide or an apoptotic fragment thereof to a cell of the mammal. The transgene is positioned for expression in the cell, and preferably encodes E4orf6.

In a third aspect, the invention provides a method of increasing apoptosis in a cell which includes administering to the cell a compound which increases E4orf6 biological activity. In various preferred embodiments, the compound is E4orf6 mRNA, or increases the stability of E4orf6.

In a preferred embodiment of the first and third aspects of the invention, the cell is in a mammal, preferably a human.

In a fourth aspect, the invention provides a method of increasing apoptosis in a

cell by administering to the cell an apoptosis inducing amount of an E4orf4 polypeptide or an apoptotic fragment thereof. In a preferred embodiment of this aspect, the apoptosis is p53-independent.

5 In a fifth aspect, the invention provides a method of increasing apoptosis in a mammal which includes providing a transgene encoding an E4orf4 polypeptide or an apoptotic fragment thereof to a cell of the mammal. The transgene is positioned for expression in the cell, and preferably encodes E4orf4.

10 In a sixth aspect, the invention provides a method of increasing apoptosis in a cell which includes administering to the cell a compound which increases E4orf4 biological activity. In various preferred embodiments, the compound is E4orf4 mRNA, or increases the stability of E4orf4.

In a preferred embodiment of the fourth and sixth aspects of the invention, the cell is in a mammal, preferably a human.

15 In a seventh aspect, the invention provides a method of increasing apoptosis in a cell by administering to the cell an apoptosis inducing amount of a composition which includes an E4orf6 polypeptide or an apoptotic fragment thereof and an E4orf4 polypeptide or an apoptotic fragment thereof. In a preferred embodiment of this aspect, the apoptosis is p53-independent.

20 In an eighth aspect, the invention provides a method of increasing apoptosis in a mammal which includes providing a first transgene encoding an E4orf6 polypeptide or fragment thereof and a second transgene encoding an E4orf4 polypeptide or fragment thereof to a cell of the mammal. The first and second transgenes are positioned for expression in the cell and, preferably, encode E4orf6 and E4orf4, respectively.

25 In a ninth aspect, the invention provides a method of increasing apoptosis in a cell which includes administering to the cell a composition which includes a first compound which increases E4orf6 biological activity and a second compound which increases E4orf4 biological activity. In various preferred embodiments, the first

compound is E4orf6 mRNA, or increases stability of E4orf6, and the second compound is E4orf4 mRNA or increases stability of E4orf4.

In a preferred embodiment of the seventh and ninth aspects of the invention, the cell is in a mammal, preferably a human.

5 In preferred embodiments of all the above aspects of the invention, the cell is in a mammal diagnosed as having a disease involving insufficient apoptosis. Preferably, the disease is cancer.

In a tenth aspect, the invention features a pharmaceutical composition which includes substantially pure nucleic acid encoding an E4orf6 polypeptide and a
10 pharmaceutically acceptable carrier. In one embodiment of this aspect, the nucleic acid encodes E4orf6 having a conservative amino acid substitution relative to the E4orf6 sequence of Fig. 15 (SEQ ID NO.: 2).

In an eleventh aspect, the invention features a pharmaceutical composition which includes nucleic acid encoding an apoptotic fragment of E4orf6.

15 In a preferred embodiment of the tenth and eleventh aspects of the invention, the nucleic acid is in a viral vector. In another embodiment, the nucleic acid is operably linked to regulatory sequences for expression of the polypeptide and the regulatory sequences include a promoter. In another embodiment, the promoter is a constitutive promoter, is inducible by one or more external agents, or is cell-type
20 specific.

In a twelfth aspect, the invention features a pharmaceutical composition which includes a nucleic acid having the sequence of Fig. 15 (SEQ ID NO.: 1), or degenerate variants thereof, and encoding the amino acid sequence of Fig. 15 (SEQ ID NO.: 2).

25 In a thirteenth aspect, the invention features a pharmaceutical composition which includes nucleic acid having about 50% or greater nucleotide sequence identity to the DNA sequence of Fig. 15 (SEQ ID NO.: 1), where the nucleic acid encodes a polypeptide with E4orf6 apoptotic biological activity. In one embodiment of this

aspect of the invention, the nucleotide sequence identity is 75% or greater to the DNA sequence of Fig. 15 (SEQ ID NO.: 1).

In a fourteen aspect, the invention features a pharmaceutical composition which includes a DNA sequence substantially identical to the DNA sequence of Fig. 15
5 (SEQ ID NO.: 1).

In a fifteenth aspect, the invention features pharmaceutical composition which includes substantially pure mammalian E4orf6 polypeptide, or apoptotic fragment thereof. In one embodiment of this aspect, the polypopetide includes an amino acid sequence substantially identical to the amino acid sequence shown in Fig. 15 (SEQ ID
10 NO.: 2). In another embodiment, the polypeptide has a conservative amino acid substitution relative to the E4orf6 sequence of Fig. 15 (SEQ ID NO.: 2).

In a sixteenth aspect, the invention features a pharmaceutical composition which includes a substantially pure polypeptide fragment of E4orf6.

In a seventeenth aspect, the invention features a pharmaceutical composition
15 which includes substantially pure nucleic acid encoding an E4orf4 polypeptide and a pharmaceutically acceptable carrier. In one embodiment of this aspect, the nucleic acid encodes E4orf4 having a conservative amino acid substitution relative to the E4orf4 sequence of Fig. 16 (SEQ ID NO.: 4).

In an eighteenth aspect, the invention features a pharmaceutical composition
20 which includes nucleic acid encoding an apoptotic fragment of E4orf4.

In a preferred embodiment of the seventeenth and eighteenth aspects of the invention, the nucleic acid is in a viral vector. In another embodiment, the nucleic acid is operably linked to regulatory sequences for expression of the polypeptide and the regulatory sequences include a promoter. In another embodiment, the promoter is
25 a constitutive promoter, is inducible by one or more external agents, or is cell-type specific.

In a nineteenth aspect, the invention features a pharmaceutical composition which includes a nucleic acid having the sequence of Fig. 16 (SEQ ID NO.: 3), or

degenerate variants thereof, and encoding the amino acid sequence of Fig. 16 (SEQ ID NO.: 4).

5 In a twentieth aspect, the invention features a pharmaceutical composition which includes nucleic acid having about 50% or greater nucleotide sequence identity to the DNA sequence of Fig. 16 (SEQ ID NO.: 3), where the nucleic acid encodes a polypeptide with E4orf4 apoptotic biological activity. In one embodiment of this aspect of the invention, the nucleotide sequence identity is 75% or greater to the DNA sequence of Fig. 16 (SEQ ID NO.: 3).

10 In a twenty-first aspect, the invention features a pharmaceutical composition which includes a DNA sequence substantially identical to the DNA sequence of Fig. 16 (SEQ ID NO.: 3).

In a twenty-second aspect, the invention features pharmaceutical composition which includes substantially pure mammalian E4orf4 polypeptide, or apoptotic fragment thereof. In one embodiment of this aspect, the polypeptide includes an amino acid sequence substantially identical to the amino acid sequence shown in Fig. 16 (SEQ ID NO.: 4). In another embodiment, the polypeptide has a conservative amino acid substitution relative to the E4orf4 sequence of Fig. 16 (SEQ ID NO.: 4).

15 In a twenty-third aspect, the invention features a pharmaceutical composition which includes a substantially pure polypeptide fragment of E4orf4.

20 In the twenty-fourth and twenty-fifth aspects, the invention features methods for identifying a compound as an E4orf6 analog or an E4orf4 analog which includes first providing a cell expressing the adenovirus E1A-289R protein while not expressing any E4 proteins. The cell is then contacted with a candidate compound and cell viability is determined, where death in the cell indicates a compound that is an E4orf6 or an E4orf4 analog.

25 In one embodiment of the twenty-fourth and twenty-fifth aspects of the invention, the cell is selected from the group consisting of: 1A.A3, 1A.A6, and 1A.A12 cells. In other preferred embodiments, the viability is measured with Trypan

Blue™, a DNA fragmentation assay, an Annexin V binding assay, or Propidium Iodide, or a combination thereof. In yet another embodiment, the cell is infected with a mutant adenovirus incapable of expressing any E4 proteins.

5 In a twenty-sixth aspect, the invention features a method for identifying a compound as an E4orf4 analog which includes first providing a cell expressing protein phosphatase 2A. The cell is then contacted with the compound the activity of the protein phosphatase 2A in the cell is measured, where an increase in the activity relative to a cell not contacted indicates a compound that is an E4orf4 analog.

10 In a twenty-seventh aspect, the invention features a pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which includes E4orf6, an analog, or a biologically active fragment thereof.

15 In a twenty-eighth aspect, the invention features a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which includes a therapeutical amount of E4orf6, an analog, or a biologically active fragment thereof in association with a pharmaceutical carrier.

20 In a twenty-ninth aspect, the invention features a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which includes a therapeutic amount of a compound which induces apoptosis or other cytotoxic effects analogous to E4orf6 biological activity in association with a pharmaceutical carrier.

In a thirtieth aspect, the invention features a pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which includes E4orf4, an analog, or a biologically active fragment thereof.

25 In a thirty-first aspect, the invention features a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which includes a therapeutic amount of E4orf4, an analog, or a biologically active fragment thereof in association with a pharmaceutical carrier.

In a thirty-second aspect, the invention features a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which includes a therapeutic amount of a compound which induces protein phosphates 2a in association with a pharmaceutical carrier. In one embodiment of this aspect, the
5 compound is an agonist of E4orf4. In another embodiment, the compound mimics E4orf4 activity.

In a thirty-third aspect, the invention features a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which includes a therapeutic amount of a compound which induces apoptosis or other
10 cytotoxic effects analogous to E4orf4 biological activity in association with a pharmaceutical carrier.

In a thirty-fourth aspect, the invention features a pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which includes E4orf6, an analog, or a biologically active
15 fragment thereof; and E4orf4, an analog, or a biologically active fragment thereof.

In a thirty-fifth aspect, the invention features a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which includes a therapeutic amount of E4orf6, an analog, or a biologically active fragment thereof; and E4orf4, an analog, or a biologically active fragment thereof in association
20 with a pharmaceutical carrier.

In a thirty-sixth aspect, the invention features a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which includes a therapeutic amount of a compound which induces apoptosis or other cytotoxic effects analogous to biological activities of the E4 death proteins in
25 association with a pharmaceutical carrier.

Compositions of the invention include, but are not limited to, E4orf4 protein, E4orf6 protein, combinations thereof, nucleic acids encoding the E4orf4 and E4orf6 polypeptides, analogs, mimetics, and any agonist therapeutic agents identified using

any of the methods disclosed herein. The compositions may be administered with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer such compositions to patients.

5 In accordance with the present invention, the expression "diseases which involve inappropriate cell survival" includes, without limitation, diseases caused by HIV, herpes and/or other viral infections, Alzheimer's disease, cancer, arthritis, and lupus.

10 In accordance with the present invention, "E4 death proteins" include products encoded by DNA capable of hybridizing at high stringency conditions to nucleic acids encoding E4orf6 (SEQ ID NO.: 1) and E4orf4 (SEQ ID NO.: 3), provided in Figs. 15 and 16 respectively, and which also have E4orf6 and/or E4orf4 biological activity. Products are encoded by DNA that is at least 500 nucleotides in length, preferably, less than 200 nucleotides in length, more preferably, less than 150 nucleotides in
15 length, and most preferably, less than 100 nucleotides in length. It will be understood that E4orf6 and E4orf4 proteins and nucleic acids of the invention may be obtained from any adenovirus strain having the E4orf6 or E4orf4 open reading frames, as defined as an open reading frame which is at least 20%, preferably 50%, more preferably 75%, and most preferably 90% identical to the E4orf6 (SEQ ID NO. 2) and
20 E4orf4 (SEQ ID NO.: 4) open reading frames, respectively, provided herein.

 In accordance with the present invention, "E4orf6 proteins" and "E4orf6 polypeptides" include products encoded by DNA capable of hybridizing at high stringency conditions to nucleic acids encoding E4orf6 (SEQ ID NO.: 1) provided in Fig. 15, and which also have E4orf6 biological activity. Products are encoded by
25 DNA that is at least 500 nucleotides in length, preferably, less than 200 nucleotides in length, more preferably, less than 150 nucleotides in length, and most preferably, less than 100 nucleotides in length. It will be understood that E4orf6 proteins and nucleic acids of the invention may be obtained from any adenovirus strain having the E4orf6

open reading frame, as defined as an open reading frame which is at least 20%, preferably 50%, more preferably 75%, and most preferably 90% identical to the E4orf6 open reading frame (SEQ ID NO.: 2) provided herein.

5 In accordance with the present invention, "E4orf4 proteins" and "E4orf4 polypeptides" include products encoded by DNA capable of hybridizing at high stringency conditions to nucleic acids encoding E4orf4 (SEQ ID NO.: 3), provided in Fig. 16, and which also have E4orf4 biological activity. Products are encoded by DNA that is at least 500 nucleotides in length, preferably, less than 200 nucleotides in length, more preferably, less than 150 nucleotides in length, and most preferably, less
10 than 100 nucleotides in length. It will be understood that E4orf4 proteins and nucleic acids of the invention may be obtained from any adenovirus strain having the E4orf4 open reading frame, as defined as an open reading frame which is at least 20%, preferably 50%, more preferably 75%, and most preferably 90% identical to the E4orf4 open reading frame (SEQ ID NO.: 4) provided herein.

15 In accordance with the present invention, the expression "high stringency conditions" means conditions that allow DNA hybridization to nucleic acids encoding E4orf6 (SEQ ID NO.: 1) or E4orf4 (SEQ ID NO.: 3) at high stringency (e.g., hybridizing in 2X SSC at 40°C with a DNA probe length of at least 40 nucleotides). For other definitions of high stringency conditions, see Ausubel, F. et al., 1994,
20 Current Protocols in Molecular Biology, John Wiley & Sons, New York, 6.3.1 - 6.3.6, hereby incorporated by reference.

In accordance with the present invention, the expression "E4orf6 biological activity" means the ability to induce in E1A-289R expressing cells an increased cell death that is 25%, more preferably 40%, and most preferably 60% greater than the cell
25 death observed in E1A-289R expressing cells not expressing E4orf6, an apoptotic fragment, or analog thereof. E4orf6 biological activity is determined using one of the assays provided herein, preferably the luciferase death assay using 1A.A3, 1A.A6, or 1A.A12 cells. It will be understood that E1A-289R may be from any adenovirus

strain.

In accordance with the present invention, the expression "E4orf4 biological activity" means the ability to induce in E1A-289R expressing cells an increased cell death that is 50%, more preferably 75%, and most preferably 90% greater than the cell death observed in E1A-289R expressing cells not expressing E4orf4, an apoptotic fragment, or analog thereof. E4orf6 biological activity is determined using one of the assays provided herein, preferably the luciferase death assay using 1A.A3, 1A.A6, or 1A.A12 cells. It will be understood that E1A-289R may be from any adenovirus strain.

In accordance with the present invention, the expression "promoter" means a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene.

In accordance with the present invention, the expression "degenerate variant" means nucleic acid sequences or combinations thereof selected from all possible coding sequences for E4orf6 and E4orf4, or polypeptide fragments thereof, based upon the universal genetic code.

In accordance with the present invention, the expression "operably linked" means that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences.

In accordance with the present invention, the expression "positioned for expression" means that the nucleic acid is positioned adjacent to a nucleic acid sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., an E4orf4 polypeptide, a recombinant protein or a RNA molecule).

In accordance with the present invention, the expression "substantially identical" means a polypeptide or nucleic acid exhibiting at least 50%, preferably 75%, more preferably 90%, and most preferably 95% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences
5 will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 70 nucleotides, more preferably at least 90 nucleotides, and most preferably at least 120 nucleotides.

10 Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and
15 other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "transformed cell" is meant a cell which is immortalized. For example, a
20 transformed cell will divide and give rise to two daughter cells of the same differentiation status as the parent cell. A transformed cell may be a cell into which (or into an ancestor of which) has been introduced an exogenous gene or gene product (e.g., an oncogene) which allows immortalization of that cell. A transformed cell may also arise from a genomic mutation in an endogenous gene, giving rise to a mutated
25 gene product, or dysregulation of a endogenous gene product. Transformed cells are differentiated from stem cells in that transformed cells have an alteration affecting normal gene expression and/or regulation. Exemplary transformed cells include cancerous cells such as those found in solid and liquid tumors.

In accordance with the present invention, the expression "transgene" means any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or
5 may represent a gene homologous to an endogenous gene of the organism.

In accordance with the present invention, the expression "transgenic" means any cell which includes a nucleic acid sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic cells are generally transgenic mammalian cells and the
10 nucleic acid (transgene) is inserted by artifice into the nuclear genome.

In accordance with the present invention, the expression "polypeptide" means any chain of more than two amino acids, regardless of post-translational modification, such as glycosylation or phosphorylation. Polypeptides include proteins, polypeptide fragments thereof, peptide mimetics thereof, and mutants thereof.

15 In accordance with the present invention, the expression "apoptotic fragment" means a polypeptide fragment of an E4 death protein (i.e., E4orf4 and E4orf6) that has a transformed cell-killing ability that is 75%, more preferably 95%, or most preferably, 100% or greater when compared to the transformed cell-killing ability of the full length protein.

20 In accordance with the present invention, the expression "substantially pure polypeptide" means a polypeptide that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is an E4
25 death protein polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure E4 death protein polypeptide may be obtained, for example, by extraction from a natural source (e.g. an adenovirus) by expression of a recombinant nucleic acid encoding an E4 death protein

polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

5 A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from adenoviruses but synthesized in *E. coli* or other
10 prokaryotes. By "substantially pure nucleic acid" is meant nucleic acid (e.g., DNA) that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote
15 or eukaryote; or which exists as a separate molecule (e.g., a DNA or a DNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

In accordance with the present invention, the expression "specifically binds" is
20 meant an antibody that recognizes and binds a protein but that does not substantially recognize and bind other molecules in a sample, e.g., a biological sample, that naturally includes protein. Preferably, the specifically binding antibody which specifically binds an adenovirus protein (e.g., E4orf4) does not bind another adenovirus protein (e.g., E4orf6).

25 In accordance with the present invention, the expression "analog" includes, without limitation, polypeptide fragments, peptide and non-peptide mimetics, reagents and compounds which mimic the cell killing function, and reagents and compounds which mimic other functions of E4orf4 or E4orf6 proteins.

In accordance with the present invention, the expression "pharmaceutically acceptable carrier" means a carrier which is physiologically acceptable to the treated mammal while retaining the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline. Other physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in Remington's Pharmaceutical Sciences, (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA.

In accordance with the present invention, the expression "viral vector" means a strand of DNA which includes elements taken from a virus. Viral vectors may direct the expression of inserted DNA from an exogenous promoter, or may direct expression of inserted DNA from the vector's own long terminal repeat (LTR) sequences. Preferable viral vectors are able to be packaged in non-lytic viruses capable of infecting cells which then expressed the DNA inserted into the viral vector.

The pharmaceutical agent of the present invention allows for the selective killing of cells that are prevented from dying by a virus or as a consequence of a disease state. Thus, the pharmaceutical agent of the present invention only kills the inappropriately surviving cells, such as cancer cells or viral infected cells. This results in a substantially side effect free therapy for the patient.

The pharmaceutical agent of the present invention includes, without limitation, E4 death proteins of any adenovirus of any serotype, fragment thereof, and peptide and non-peptide mimetics of these protein products.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A shows the regions encoding amino acid sequences of the Ad5 E1A proteins and mutants thereof;

Fig. 1B summarizes relevant adenovirus mutants;

Fig. 2 is an agarose gel analysis of the induction of DNA fragmentation by indicated Ad5 mutants in p53-null 10(1) cells;

5 Figs. 3A and 3B are graphs of the Trypan Blue™ exclusion analyses showing viability of p53-null Saos-2 cells (Fig. 3A) and p53-null Saos-2 cells expressing Bcl-2 (Fig. 3B) infected with indicated Ad5 mutants;

Fig. 4 is an agarose gel analysis of the induction of DNA fragmentation by indicated E1A mutants in p53-null 10(1) cells;

10 Fig. 5 is a graph of the Trypan Blue™ exclusion analysis showing viability of p53-null Saos-2 cells infected with indicated E1A mutants;

Fig. 6 is an agarose gel analysis of the induction of DNA fragmentation by indicated Ad5 mutants in E1A/MEF and Hy/MEF cells;

Fig. 7 is a graph of the Trypan Blue™ exclusion analysis showing viability of p53-null Saos-2 cells infected with indicated Ad5 mutants;

15 Fig. 8 is an agarose gel analysis of the induction of DNA fragmentation by indicated Ad5 mutants in Hy/MEF and E1A/MEF cells;

Figs. 9A and 9B are graphs of the Trypan Blue™ exclusion analyses showing E1A/MEF (Fig. 9A) and Hy/MEF (Fig. 9B) cell killing by indicated Ad5 mutants;

20 Fig 10 shows the regions encoding amino acid sequences of the E4orf proteins and indicates E4orf expression in Ad5 mutants;

Fig. 11 is a graph of the Trypan Blue™ exclusion analysis showing the role of E4orf6 in p53-independent cell killing (in p53-null Saos-2 cells);

Fig. 12 is a graph of the Trypan Blue™ exclusion analysis showing the role of E4orf4 in p53-independent cell killing (in p53-null Saos-2 cells);

25 Figs. 13A and 13B are graphs showing that E4orf4 or E4orf6, when expressed by transient transfection of an E4orf4- or an E4orf6-encoding plasmid, induces cytotoxicity in p53-null cells, as judged by the low expression of a co-transfected reporter plasmid encoding luciferase, relative to non-cytotoxic inducing control

plasmids encoding E4orf1, E4orf3, and crmA;

Fig. 14 is a graph showing that E4orf4 and E4orf6, when expressed individually or together with luciferase by transient transfection of an E4orf4- or an E4orf6-encoding plasmid induces cytotoxicity in p53-null cells, as judged by the low expression of a co-transfected reporter plasmid encoding luciferase, relative to non-cytotoxic inducing control plasmids encoding E4orf1, E4orf2, and E4orf3. Two concentrations of the E4orf plasmid DNAs were used: black bars indicate 2.5 μ g; cross-hatched bars indicate 5 μ g;

Fig. 15 shows the nucleic acid (top) (SEQ ID NO.: 1) and amino acid (bottom) (SEQ ID NO.: 2) sequences of Adenovirus type 5 (Ad5) E4orf6; and

Fig. 16 shows the nucleic acid (top) (SEQ ID NO.: 3) and amino acid (bottom) (SEQ ID NO.: 4) sequences of Adenovirus type 5 (Ad5) E4orf4.

DETAILED DESCRIPTION OF THE INVENTION

We have discovered that in the absence of E1B, E1A open reading frame (orf) protein products also induce p53-independent apoptosis. Our results indicate that such apoptotic cell death is only induced by the 289R E1A protein. Furthermore, when p53-null mouse cells constitutively expressing E1A products are infected by an adenovirus vector lacking the entire E1A and E1B coding regions but containing early regions E2, E3 and E4, rapid cell death due to apoptosis is observed. Furthermore, we show that 289R induces apoptosis in p53-null mouse and human cells, and that such p53-independent cell death requires the expression of another early viral gene. In the present invention, we describe genetic analyses which indicate that neither E2 nor E3 products are necessary for p53-independent cell death and that two E4 proteins are responsible for p53-independent cell death induced by the 289R E1A product. These results indicate that the role of E1A-289R may be to transactivate expression of an additional early transcript whose product actually induces p53-independent apoptosis. We provide herein that two E4 gene products are responsible for such cell killing and

therapeutic compositions, methods, and drug screens which stem from this discovery.

We show that both the E1B-19kDa protein and cellular Bcl-2 inhibit or significantly delay p53-independent apoptosis. Neither early regions E2 or E3 appear to be necessary for such cell death. Analysis of a series of E1A mutants indicates that mutations in the transactivation domain and other regions of E1A correlate with E1A-mediated transactivation of E4 gene expression. Furthermore, p53-deficient human Saos-2 cells infected with a mutant adenovirus which expresses E1B but none of the E4 gene products remains viable for a considerably longer period of time than p53-deficient human Saos-2 cells infected with wild-type (wt) Ad5. In addition, an adenovirus vector lacking both E1 and E4 is unable to induce DNA degradation and cell killing in E1A-expressing cell lines. These data showed that an E4 product was essential for E1A-induced p53-independent apoptosis.

The following examples are meant to illustrate the invention and do not limit the invention.

I. Materials and Methods Used in the Present Invention.

Cells and viruses

Human Saos-2 cells (ATCC HTB 85) and 10(1) mouse embryo fibroblast-derived cells, which are both deficient for p53 expression, were cultured in 60 mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in Dulbecco's modified MEM (DMEM) supplemented with 10% fetal calf serum (FCS) as were both NIH-3T3 and CHO cells. The cell line Saos-2/Bcl-2(3g4), which stably expresses Bcl-2, was derived for this study from Saos-2 cells by selection with G418, as was the control line Saos-2/Neo(2a2). 1A.A3, 1A.A6 and 1A.A12 mouse embryo fibroblast (MEF) cell lines expressing Ad5 E1A proteins, and Hy.A3 hygromycin-selected control lines, have been described previously (Lowe, S.W. et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91: 2026-2030), and were cultured in DMEM containing 10% FCS and 100 mg/mL of hygromycin. Normally cells were infected with mutant or

wild-type (wt) Ad5 at a multiplicity of 100 pfu per cell. Ad5 E1A mutants are illustrated in Fig. 1A and include deletion mutants *dl1101* (residues 4-25 deleted), *dl1143* (38-60), *dl1107* (111-123), *dl1108* (124-127), *dl1143/08* (38-60 plus 124-127) and *dl1132* (224-238). Proteins encoded by some of the mutants used in the present studies have been presented, including the residues removed in deletion mutants. CR1, CR2, CR3, AR1 and AR2 have also been indicated. A new E1A mutant termed AR1⁻/E1B⁻, which lacks the entire AR1 region (residues 189-200) and also fails to express E1B products, was generated following the methods previously described in Dumont, D. J. et al. (J. Virol. 63: 987-991, 1989). Further mutants were generated according to standard molecular biology techniques. Mutant AR2⁻/E1B⁻ was generated by introducing *dl1132*, which lacks residues 224-238, into a background that fails to express E1B proteins. Mutant AR1⁻/AR2⁻/E1B⁻ represents a combination of the latter two mutants. Additional E1A mutants containing single amino acid substitutions at various sites within CR3 were produced by subcloning appropriate restriction enzyme fragments from mutant E1A cDNA plasmids into genomic viral DNA, followed by rescue into virus to form mutants AD147VL (Val-147 converted to Leu), AD177CS, and AD185SG. All other mutants have been summarized in Fig. 1B, which is a list providing the names and defects of E1B and other mutants. Two mutants fail to express E1B proteins of 19kDa (originally termed pm1716/2072 but now called E1B/19K⁻) and 55kDa (originally pm2019/2250, now E1B/55K⁻) (McLorie, W. et al., *supra*). Mutant 12S/E1B⁻ (originally *dl520E1B⁻*) produces only the E1A-243R protein encoded by the 12S mRNA and no E1B products. Mutant E1B⁻ which expresses both major E1A products but neither the 19kDa or 55kDa E1B species was described previously (Teodoro, J.G. et al., 1995, Oncogene 11: 467-474). A mutant that expresses only 289R in the absence of E1B, termed 13S/E1B⁻, was similarly prepared for the present studies. A series of E1A mutants (*dl1101/E1B⁻*, *dl1107/E1B⁻*, AD147VL/E1B⁻, etc.), which express no E1B products, was also produced by introducing E1A mutations into mutant E1B⁻, which expresses both 289R

and 243R E1A products but no E1B (Teodoro, J.G. et al., 1995, *Oncogene* 11: 467-474). The presence of mutations in all mutants was confirmed by DNA sequencing, restriction enzyme digestion, or Southern blotting, all of which are standard molecular biology techniques. Further Ad5 vectors used in this study

5 included AdLacZ in which the E1 (E1A + E1B) region was replaced with the *E. coli* gene lacZ under the CMV promoter, and Ad5dl70-8 (F.L. Graham, McMaster University, Hamilton, Ont., Canada), which was generated by cotransfection of plasmids pAB7 and pBHG10, as described previously (Bett, A.J., 1994, *Proc. Natl. Acad. Sci. USA*, 91:8802-8806), and which lacks both E1 and the entire E3 region.

10 These viral vectors and other E1A and E1B mutants were grown on human 293 cells, as has been previously described (Graham, F. L. et al., 1977, *J. Gen. Virol.* 36: 59-72; Bett, A.J., 1994, *Proc. Natl. Acad. Sci. USA*, 91:8802-8806). Adenovirus vector AdRSV β gal.11, which lacks the entire E1 and E4 regions, was a gift of Douglas Brough (GenVec, Rockville, MD). In addition, some experiments were carried out

15 with human adenovirus type 2 (Ad2) mutant dl1019 which contains deletions that eliminate expression of all E4 products and which was propagated on W162 monkey cells, as described previously (Bridge, E. et al., 1989, *J. Virol.* 63: 631-638). Other E4 mutants (Bridge, E. et al., *supra*) have been summarized in Fig. 10.

DNA fragmentation

20 Low molecular weight DNA was isolated from mock- or Ad5-infected cells as described in Teodoro et al. (*Oncogene* 11:467-474, 1995). For these experiments, 60 mm-diameter plates of cells were harvested at 40 hours post-infection and lysed in pronase lysis buffer (10mM Tris-HCl (pH 8) containing 5mM EDTA, 100mM NaCl, and 1 mg/mL (w/v) pronase to which SDS was added to 0.5% w/v). Cell lysates were

25 next incubated at 37°C for 2 hours and NaCl was then added to a final concentration of 1M. Samples were then incubated overnight at 4°C and centrifuged at 15,000 x g for 30 min. Extracted nucleic acids were treated with RNAase A and analyzed on 1% agarose gels stained with ethidium bromide.

Cell viability assays

Cells were infected with wt or mutant virus in 24-well plates containing cells at about 80% confluence. At various times following infection adherent and non-adherent cells were pooled and viability was assessed by Trypan Blue™ exclusion. At least 300 cells were counted at each time point.

Measurement of E1A-mediated transactivation of the adenovirus E4 promoter

CAT transactivation assays were performed using NIH 3T3 or CHO cells plated at a density of 2×10^5 cells on 60 mm-diameter dishes. The E4 CAT reporter plasmid (E4-CAT) contained the E4 promoter upstream of the chloramphenicol acetyltransferase (CAT) gene. Transient co-transfections were performed by the calcium phosphate precipitation method (described, for example, in Ausubel, F. et al., 1994, Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY) using 2.5 µg of reporter plasmid DNA and 2.5 µg of DNA from plasmids expressing wt or mutant E1A products. Cells were glycerol shocked 12 hours after transfection and then harvested 36 hours later. CAT assays were performed using cell extracts containing equal amounts of β-galactosidase activity, as has been described (Ausubel, F. et al., *supra*). The amount of activity was quantified from TLC plates using a Fujix Bas 2000™ Phosphorimager.

Luciferase Death Assay

The E4 open reading frames (orfs) were individually cloned into the pCDNA3.1 plasmid (commercially available from Invitrogen) which drives gene expression with the CMV promoter. The plasmids were purified using 2X CsCl purification and transfections were done by calcium phosphate precipitation. Each E4orf-encoding plasmid was transfected together with a plasmid expressing the luciferase gene under the control of the RSV promoter (pRSV-luciferase). Either the empty pCDNA vector or a pCDNA vector encoding crmA was used as a negative control for cell killing.

II. E1A-induced p53-independent Apoptosis is Inhibited by Both the E1B-19kDa Protein and Cellular Bcl-2.

We have recently shown that whereas both major Ad5 E1A products could induce apoptosis in cells expressing p53, only the 289R E1A protein could do so in cells lacking p53 (Teodoro, J.G. et al., 1995, *Oncogene* 11: 467-474). Fig. 2 shows the pattern of DNA fragmentation in p53⁻ mouse 10(1) cells infected by various Ad5 mutants. 10(1) cells, which fail to express p53, were infected with various Ad5 mutants, or they were mock-infected, and at 40 hours post-infection (p.i.), low molecular weight DNA was analyzed by agarose gel electrophoresis. The contents of individual lanes are as indicated in Fig. 2. Extracts from mock-infected cells (Fig. 2, lane 1) and those infected with wt Ad5 (Fig. 2, lane 2), which expresses E1B products, displayed reduced levels of extracted low molecular weight DNA and little or no degraded DNA, as did those from cells infected with mutant E1B/55K⁻ (Fig. 2, lane 4), which produces the E1B-19kDa protein but not the E1B-55kDa product. With cells infected with mutant E1B⁻, which synthesizes both the 289R and 243R E1A proteins but which produces no E1B products (Fig. 2, lane 6), large amounts of DNA were extracted and high levels of nucleosome-sized DNA fragments were evident. Similar results were also obtained with cells infected with E1B/19K⁻ (Fig. 2, lane 5), which produces the E1B-55kDa species but not the 19kDa protein. Induction of DNA degradation in these p53⁻ cells did not occur following infection with 12S/E1B⁻ (Fig. 2, lane 3), which produces only E1A-243R and no E1B, but it did occur with 13S/E1B⁻ (Fig. 2, lane 7), which yields only E1A-289R in the absence of E1B products. Thus, E1A-289R, but not 243R, induced p53-independent apoptosis in the absence of E1B proteins. In addition, these results indicated that the E1B 19kDa polypeptide but not the 55kDa E1B product was able to protect against apoptosis induced by E1A in the absence of p53.

To examine the specificity of inhibition of apoptosis further, studies were conducted to determine if the cellular Bcl-2 protein was also able to prevent

p53-independent apoptosis as several previous studies had shown that Bcl-2 and the E1B-19kDa protein may be functionally similar (Nguyen, M. et al., 1994, J. Biol. Chem. 269: 16521-16524). Human Saos-2 cells, which are defective for synthesis of p53, were transfected with cDNAs encoding the human Bcl-2 protein and the
5 neomycin resistance marker and several cell lines were selected using G418. One such Bcl-2 expressing clone, termed Saos-2/Bcl-2(3g4), and a control Saos-2 clone, Saos-2/neo(2a2) selected only for resistance to G418, were infected with wt Ad5, mutants 12S/E1B⁻ or E1B/19K⁻, or were mock-infected, and cell viability assays were conducted at various times after infection. P53-deficient human Saos-2/neo(2a2) cells
10 (Fig. 3A) or Saos-2/Bcl-2(3g4), which express human Bcl-2 constitutively (Fig. 3B), were mock-infected or infected with wt, E1B/19K⁻ or 12S/E1B⁻ and were tested for viability by a Trypan Blue™ exclusion assay at various times following infection. Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in Figs. 3A and 3B. Fig. 3A shows that Saos-2/neo(2a2) control cells were
15 killed by the E1B/19K⁻ virus that expresses E1A-289R, but those infected with wt or 12S/ E1B⁻ remained almost as viable as mock-infected cells during the test period. Fig. 3B shows that with Saos-2/Bcl-2(3g4) cells which stably express high levels of Bcl-2, little cell death was induced by the E1B/19K⁻ virus. Similar results were obtained with three other control and Bcl-2 producing Saos-2 cell lines. Thus, like the
20 E1B-19kDa protein, Bcl-2 also blocks E1A-induced p53-independent apoptosis.

III. Role of E1A Domains in p53-independent Apoptosis.

To investigate the regions of E1A products involved in causing p53-independent cell death, p53⁻ mouse 10(1) cells were infected with Ad5 mutants which fail to express E1B and which harbor a variety of defects at various regions of
25 the E1A molecule. Extracts were harvested and analyzed on gels to determine the extent of degradation of low molecular weight DNA. An experiment in p53-deficient 10(1) cells was performed using a series of Ad5 E1A mutants defective in expression

of E1B products. 10(1) cells were infected with various Ad5 mutants, or they were mock-infected, and at 40 hours p.i., low molecular weight DNA was analyzed by agarose gel electrophoresis. The contents of individual lanes are as indicated in Fig. 4. Fig. 4 again shows that mutant E1B/19K⁻ (Fig. 4, lane 3) induced DNA degradation whereas such DNA degradation did not occur with wt Ad5 (Fig. 4, lane 2) or mock-infected cells (Fig. 4, lane 1). Mutants which affected the E1A transactivation function associated with CR3 all failed to induce DNA degradation. These included 12S/E1B⁻ (Fig. 4, lane 8), and point mutants AD147VL/E1B⁻, AD171CS/E1B⁻ and AD185SG/E1B⁻ (Fig. 4, lanes 9 to 11, respectively) which carry single residue substitutions at critical residues in CR3 that eliminate E1A transactivation activity. In addition, deletion of AR1 or both AR1 and AR2 (AR1⁻/E1B⁻ and AR1⁻/AR2⁻/E1B⁻ in Fig. 4, lanes 12 and 14, respectively) also eliminated DNA degradation whereas removal of AR2 alone (AR2⁻/E1B⁻ in Fig. 4, lane 13) had little effect on eliminating DNA degradation. Interestingly, mutants in CR2 which eliminate complex formation with pRB and related proteins (*d*11107/E1B⁻ and *d*11108/E1B⁻ in Fig. 4, lanes 5 and 6, respectively) had no effect on the induction of DNA degradation, whereas those that eliminated binding of p300 by removal of the N-terminus (*d*11101/E1B⁻ in Fig. 4, lane 4) or a portion of CR1 as well as the pRB binding site (*d*11143/08/E1B⁻ in Fig. 4, lane 7) no longer caused this DNA degradation effect. These results suggested that E1A-induced p53-independent apoptosis required the CR3 transactivation domain, AR1, and the regions necessary for binding of p300 but not pRB-related proteins. Fig. 5 shows that similar results were obtained with these mutants in cell killing assays. An experiment was carried out in which Saos-2 cells were infected with various adenovirus mutants or mock-infected and were then tested for viability by a Trypan Blue™ exclusion assay at various times following infection. Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in Fig. 5. Cell death was induced by the E1B/19K⁻ virus, which expresses both E1A products, and by *d*11107/E1B⁻. Mutant AR2⁻/E1B⁻, which lacks AR2, also killed, but

was consistently less toxic than the former viruses. All other mutants affecting CR3, AR1 and the p300 binding sites failed to kill significantly during the test period.

IV. Activation of E4 Expression and Apoptosis.

Studies were carried out to examine the pattern of E1A transactivation of the E4 promoter in which plasmid DNA encoding various mutants forms of E1A-289R was co-transfected into NIH-3T3 or CHO cells along with DNA from E4-CAT, a construct that encodes CAT under the control of the Ad5 E4 promoter. Table 1 shows that in addition to CR3, activation of the E4 promoter required AR1 and to some extent AR2.

Table 1

E4 Transactivation by E1A Mutants

E1A Mutant	Mutation	Region Affected	E4 CAT *Activity (% wt+/-S.D.)
wt	none	none	100
<i>dl1101</i>	Δ 4-25	N-terminus	30+/-11
15 <i>dl1104</i>	Δ 48-60	CR1	40+/-5
<i>dl1107</i>	Δ 111-123	CR2	85+/-5
<i>dl1108</i>	Δ 124-127	CR2	81+/-14
<i>dl520</i>	Δ 140-185	CR3	10+/-7
AR1	Δ 189-200	AR1	25+/-7
20 AR2(pm1132)	Δ 224-238	AR2	64+/-16

* CHO or 3T3 cells were transfected with plasmid DNA encoding various E1A mutants and CAT under the Ad5 E4 promoter.

Cell extracts were assayed for CAT activity, as described herein. The CAT activity has been expressed as a percentage of that obtained with wt. Three independent assays were done for each mutant.

In addition, regions at the N-terminus and in the CR1 involved in binding of p300 were also found to be of importance in the activation of the E4 promoter. These results closely paralleled the pattern of E1A-induced p53-independent apoptosis and suggested that E4 products might be involved in this p53-independent cell death.

5 **V. E2 and E3 Products Are Not Required for Apoptosis.**

Given the results described herein outlining the requirements for the E1A-induction of p53-independent apoptosis, we determined that it was unlikely that E2 products were responsible for the induction of p53-independent apoptosis. First of all, in addition to CR3, complex formation involving CR2 and the pRB family of
10 proteins activates E2 expression, and CR2 was shown to be of little importance in cell killing. Secondly, reasonably high levels of expression of E2 proteins are known to be induced by the E1A-243R protein which is completely unable to induce p53-independent apoptosis.

Experiments were next carried out to determine if any E3 products were
15 involved in the induction of p53-independent apoptosis. The 1A.A3 mouse embryo fibroblast cell line lacking p53 but expressing Ad5 E1A proteins, and Hy.A3 hygromycin-selected p53⁻ control cells (Lowe, S.W. et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91: 2026-2030), were infected with wt Ad5, the E1B/19K⁻ virus, adenovirus vector AdLacZ, which contains lacZ in place of E1A and E1B, or with vector
20 Ad5dl70-8, which lacks both the entire E1 and E3 regions. Cell extracts were then assayed for the presence of degraded DNA.

Accordingly, cell lines expressing 289R and 243R E1A proteins constitutively (E1A/MEF), or the Hy.A3 (HY/MEF) non-expressing control cell line, were mock-infected or infected with wt Ad5 or adenovirus vectors AdLacZ or Ad5dl70-8.
25 After 40 hours, DNA was extracted and analyzed by agarose gel electrophoresis. The contents of individual lanes are as indicated in Fig. 6. Fig. 6 shows that high levels of DNA degradation were induced in 1A.A3 cells with the 19K⁻ mutants as well as both

adenovirus vectors AdLacZ or Ad5 Δ 170-8. Fig. 6 also shows that in the control cells lacking constitutive E1A expression, only the E1B/19K⁻ virus induced DNA degradation. Similar results were also obtained with two other similar E1A-expressing cell lines, 1A.A6 and 1A.A12. These results indicated that E3 products were not required for induction of p53-independent apoptosis by E1A under these conditions.

VI. E4 proteins are Essential for p53-independent Apoptosis.

To determine directly if E4 products were involved in the induction of cell death, as suggested by experiments described above, two approaches were taken. In the first approach, human p53⁻ Saos-2 cells were infected with wt Ad5 or wt adenovirus type 2 (Ad2), or with Ad2 mutant Δ 11019 which produces no E4 proteins (Bridge, E. et al., *supra*), or they were mock-infected. Although these viruses express E1B proteins and thus are protected from E1A-induced apoptosis, it was thought that if E4 products were essential for p53-independent cell death, some difference in long term cell survival might be observed. Thus, at various times up to 10 days, infected cultures were tested for cell viability. For this experiment, Saos-2 cells were mock-infected or infected with wt Ad5 or Ad2, or Δ 11019. At various times up to 10 days cell viability was assessed by Trypan BlueTM exclusion. Data have been expressed as % cell viability and symbols are as indicated in Fig. 7. Fig. 7 shows that cells infected by wt Ad5 virus began to die at about 100 hours p.i., and by 240 hours p.i. almost all of the cells were dead. Similar results were obtained with wt Ad2 (adenovirus type 2) infected cells. However, such was not the case with Δ 11019-infected cells, which remained almost as viable as mock-infected cells even 10 days after infection. These results indicated that an E4 product was involved in cell killing in the absence of p53. This idea was confirmed in experiments involving infection of E1A-expressing p53⁻ 1A.A3 cells with the adenovirus vector AdRSV β gal.11 in which both the E1 and E4 regions had been completely deleted.

For this experiment, cell lines expressing 289R and 243R E1A proteins constitutively, or the Hy.A3 non-expresser control cell line, were mock-infected or infected with wt Ad5, E1B/19K⁻, 12S/E1B⁻, or the adenovirus vector AdRSVβgal.11, which lacks both E1 and E4. After 40 hours, DNA was extracted and analyzed by agarose gel electrophoresis. The contents of individual lanes are as indicated in Fig. 8. Fig. 8 shows that in control Hy.A3 (Hy/MEF) p53⁻ cells, which do not express E1A, only the E1B/19K⁻ Ad5 mutant caused DNA degradation, and neither wt, 12S/E1B⁻, nor the AdRSVβgal.11 vector had any significant effect (i.e., did not induce DNA degradation). With 1A.A3 cells (E1A/MEF), both the E1B/19K⁻ (Fig. 8, lane 8) and 12S/E1B⁻ (Fig. 8, lane 9) viruses induced DNA degradation, but the AdRSVβgal.11 vector (Fig. 8, lane 10) still had little effect on the induction of DNA degradation. Similar results were obtained with the other two sister cell lines, 1A.A6 and 1A.A12. The ability of the E1B/19K⁻ and 12S/E1B⁻ viruses to induce apoptosis in 1A.A3 cells was analyzed further in cell killing experiments. Cell lines expressing 289R and 243R E1A proteins constitutively (E1A/MEF--right panel), or the Hy.A3 (Hy/MEF) non-E1A expressing control cell line (left panel), were mock-infected or infected with wt Ad5, E1B/19K⁻, 12S/E1B⁻, or the adenovirus vector AdRSVβgal.11. At various times after infection, cell viability was assessed by Trypan Blue™ exclusion. Data have been expressed as % cell viability and symbols are as indicated in the Figs. 9A and 9B. Fig. 9B shows that in the Hy.A3 (HY/MEF) control cells, only the E1B/19K⁻ virus induced cell death, whereas in 1A.A3 cells (E1A/MEF--shown in Fig. 9A), both the E1B/19K⁻ and 12S/E1B⁻ viruses did so. However, in both cases the AdRSVβgal.11-infected cells remained as fully viable as mock-infected cultures. These data thus confirmed that an E4 product was responsible for E1A-induced p53-independent cell death.

VII. Identification of Adenovirus E4 Death Proteins.

To identify which E4 product is responsible for induction of E1A-dependent

p53-independent apoptosis, p53-null mouse 10(1) cells were infected with wt Ad5 or with mutants carrying deletions in various portions of the E4 region (see Fig. 10). Saos-2 cells were infected with wt Ad5, Ad5 mutants, or were mock-infected, and cell viability assays using Trypan Blue™ were conducted at various times after infection.

5 Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in Figs. 11 and 12. Fig. 11 shows that Saos-2 cells infected by the wt virus began to die by about 125 hours following infection, and death was almost complete by 240 hours, as judged by Trypan Blue™ exclusion by living cells. Little cell death was observed in mock-infected cultures or in cultures infected by mutants *d/1019* and

10 *d/1011*, which lack the entire E4 region. Cell death similar to that found with wt was observed with E4 mutants *d/1013* which expresses E4orf6 and E4orf4, whereas little death occurred with mutant *d/1010* which expresses all E4 products except E4orf6 (Fig. 11). These results indicated that cell death occurred only when the E4orf6 protein was expressed and did not take place during the course of the experiment in its

15 absence. Thus, it is clear that expression of the E4orf6 protein is essential for the p53-independent apoptosis induced by the 289R E1A product.

Studies with *d/1013* left open the possibility that E4orf4 may also be involved in p53-apoptosis induced by the 389R E1A product, so additional mutants were tested. Further Trypan Blue™ exclusion experiments on Saos-2 cells following infection with

20 mutants *d/1014*, which expresses E4orf4 only, *d/1015*, which expresses E4orf4 and E4orf3, *d/1011*, which expresses no E4 products, as well as mock-infected cells, were conducted. Fig. 12 shows that indeed mutant *d/1014* which produces E4orf4 but no other E4 product killed quite effectively. These results indicated that expression of E4orf4 alone induced cell death. Mutant *d/1015* which produces only E4orf4 and

25 E4orf3 killed reproducibly less well than *d/1014*, suggesting that the E4orf3 protein may lessen the toxic effects of E4orf4.

Thus, Figs. 11 and 12 demonstrate that adenovirus Ad5 produces two proteins, E4orf6 and E4orf4, that each plays a role in the induction of p53-independent

apoptosis. This conclusion was confirmed in a second type of experiment in which either E4orf4 or E4orf6 was expressed alone in the absence of other viral proteins using transient transfection with E4orf4 or E4orf6 plasmid DNA in combination with a reporter plasmid encoding luciferase in p53 expressing or p53-null transformed cells.

5 1A.A3 and A1.A6 cells, which lack p53 but express the E1A oncogene constitutively, were co-transfected using the calcium phosphate method with a cDNA plasmid encoding luciferase operably linked to the RSV promoter (pRSV-luciferase) and either cDNA plasmids expressing various E4 products or CrmA operably linked to the CMV promoter. These constructs were generated by subcloning the cDNA
10 sequences encoding these protein products into the pCDNA3.1 expression plasmid commercially available from Invitrogen. Forty-eight hours following transfection, cells were lysed and luciferase activity was measured on a luminometer using the methods and reagents of the luciferase assay kit commercially available from Promega. The luciferase activity in Fig. 13A is represented in units of activity,
15 whereas the luciferase activity in Fig. 13B is represented a percentage of the maximal luciferase activity observed in cells co-transfected with luciferase- and E4orf3-encoding plasmids. Figs. 13A and 13B show that transfection by empty vector (EV), or cDNAs expressing E4orf1, E4orf3, or CrmA resulted in high levels of luciferase activity, indicating that cell viability was unaffected. However Figs. 13A and 13B
20 show that luciferase activity was partially reduced by expression of E4orf6 (Fig 13B), and greatly reduced by that of E4orf4 (Figs. 13A and 13B). Co-expression of both of the E4 death proteins resulted in even greater cell killing, as is shown in Fig. 14. These results provided independent evidence of the ability of both of these proteins to kill p53-null transformed cells, while at the same time demonstrating the inability of
25 E4orf1 and E4orf3 to kill these cells. Similar killing assays have yielded comparable results in p53-positive transformed cell lines and some immortalized rapidly-growing cells. In all assays of this type, E4orf4 was found to induce higher levels of killing

than E4orf6. However, it is difficult to predict whether or not the higher level of killing observed with E4orf4 is either as desirable or as specific as that obtained with E4orf6. Nonetheless, these results indicated that both E4orf4 and E4orf6 have the potential to kill rapidly growing transformed cells. Furthermore, as discussed herein, because such killing did not occur in normal cells infected by mutants in which E1A products were defective in their ability to induce unscheduled DNA synthesis and cell transformation, killing is limited to cancer cells, leaving normal cell populations relatively unaffected.

Adenovirus E1A products induce DNA degradation, rapid cell death and other hallmarks of apoptosis when expressed in the absence of E1B products, whose major role in lytic infection and transformation is to suppress E1A toxicity. Both the 289R and 243R E1A proteins are able to induce apoptosis through p53-dependent pathways.

E1A proteins also induce apoptosis in cells lacking p53 (Teodoro, J.G. et al., 1995, *Oncogene* 11: 467-474). We found that this p53-independent apoptosis was elicited only by the 289R E1A protein, and our results suggested that expression of one or more additional early viral genes regulated by E1A-289R was required for this apoptosis. The experiments described herein indicated that the E1B-55kDa protein was unable to block this effect, but both the E1B-19kDa product and the cellular suppressor of apoptosis, Bcl-2, significantly inhibited this response.

The major goal of the work leading to this invention was to identify which early viral transcription units were required to induce cell death in the absence of p53. Results obtained with E1A mutants clearly indicated that the CR3 is important in the ability of the E1A 289R product to induce p53-independent apoptosis. Furthermore, CR3-mediated transactivation activity appeared to be required, as several point mutants in CR3 which were known to eliminate transactivation of target genes were defective for induction of DNA degradation and cell killing. Of great interest were results obtained with mutants with defects outside CR3. Mutant *dl1108*, which lacks

the core binding site for pRB and related proteins, induced p53-independent apoptosis like wt. However, mutant *dl1101*, which binds pRB at reasonably normal levels but fails to bind the p300 transcriptional modulator, was totally defective in its ability to induce p53-independent apoptosis. These results suggest that interactions between p300 and 289R are essential to institute cell death pathways. Another possibility was offered by results obtained with two additional mutants with defects in the AR1 and AR2 regions encoded by the second exon of the 13S E1A mRNA. The AR1-defective mutant was unable to induce p53-independent apoptosis, and the mutant lacking AR2 was also somewhat impaired its ability to induce p53-independent apoptosis. These results corresponded exactly to the relative abilities of these mutant E1A molecules to transactivate the E4 promoter. We also found that *dl1101* was partially defective for transactivation of E4, thus indicating that not only are E4 products involved in induction of cell death, but also that interactions of 289R with p300 may reflect more a requirement for transactivation of E4 transcription than a direct role in apoptosis.

Early regions E2, E3 and E4 encode a variety of products which could play some role in cell death. E2 proteins are largely involved in viral DNA synthesis. However, it is unlikely that any E2 proteins play an essential role in cell death. First, E2 transcription requires not only CR3, but also the formation of complexes with pRB which result in the activation of the E2F family of transcription factors and E2 gene expression. Our results clearly indicated that complex formation with pRB was not essential for apoptosis. Second, the adenovirus vector AdRSV β gal.11 contained a wt E2 region and yet was defective for induction of p53-dependent apoptosis in E1A-expressing cells. The E3 region encodes several proteins which affect virus-host interactions, however, the adenovirus vector Ad5*dl70-8*, which lacks expression of E1A, E1B, and E3, was fully capable of inducing apoptosis in E1A expressing p53-deficient cells. The pattern of apoptosis we observed with E1A mutants suggested that the early viral proteins associated with cell death were encoded by E4. Direct evidence that an E4 protein was responsible was obtained from experiments in

which the pattern of death was observed in p53-null Saos-2 cells infected by wt Ad5 or a mutant defective in E4 expression. Because E1B products were expressed by these viruses, cell death occurred only at late times, but the observation that E4 mutant-infected cells displayed considerably retarded death clearly implicated an E4 product in the death process. Final confirmation came from the results of experiments with the AdRSV β gal.11 adenovirus vector which demonstrated that this viral vector was defective for cell killing. This virus was unable to induce DNA degradation or cell killing in p53-deficient cells expressing E1A.

Hence, we have shown that either of the E4 death proteins, E4orf6 or E4orf4, is responsible for the ultimate death of human cells following productive infection by adenoviruses. Cell death could be induced early after infection following expression of E1A proteins, however, p53-dependent apoptosis, which is induced directly by E1A, is blocked by expression of both the 55kDa and 19kDa E1B proteins. Following expression of E4orf4 or E4orf6 proteins, infected cells would die by p53-independent apoptosis were it not for the E1B-19kDa product which blocks cell death until late in infection. Cell death may eventually occur because the levels of the E4orf4 or E4orf6 become too elevated for suppression by E1B-19K.

The E4orf4 and E4orf6 proteins are of use in killing cells that accumulate in several disease states, including some auto-immune disorders and cancer. Such cells fail to die by apoptosis and, at least in many cancers, one reason is because many cancer cells lack or express a mutant form of p53. These cells would, however, be susceptible to killing by the E4orf4 and E4orf6 proteins.

E4orf6 is known to form complexes with E1B-55K. These complexes regulate late transport and stability of viral mRNAs and play a role in the late shut-off of host cell metabolism. We have found that E4orf6 and E1B-55K block the accumulation of p53, and that all three of these proteins interact directly. E4orf4 has been found to bind to and activate protein phosphates 2A (PP2A), which results in decreased phosphorylation of both E1A-289R and some transcription factors (Muller, U. et al.,

1992, J. Virol. 66: 5867-5878; Bondesson, M. et al., 1996, J. Virol. 70: 3844-3851).

We have also found that decreased phosphorylation of E1A probably results from the inactivation by PP2A of MAP kinase which acts on sites in CR3 required for efficient transactivation of E4.

5 The present invention allows the utilization of E4orf4 and E4orf6 adenovirus death proteins as therapeutic agents with the ability to induce apoptosis in tumor cells for the treatment of human cancers, including p53-null tumors, since these proteins are able to kill tumor cells whether p53 is present or not. Furthermore, because normal cells lack inappropriate growth signals such as those delivered by E1A or activated
10 cellular oncogenes, these E4 death proteins have little effect on normal tissues.

VIII. Additional Cell Killing Assays.

It will be understood by the skilled artisan that cell killing assays in addition to the assays already described herein may be utilized for the identification of regions of E4orf4 and E4orf6 important for cell killing, as well as the identification of other viral
15 death proteins and reagents. Additional killing assays may be developed in which the effects of the individual viral proteins can be measured in the entire cell population. Such assays are especially critical in determining the selectivity of killing by these agents. New killing assays allow a meaningful analysis of the killing potential of the E4 death proteins, fragments thereof, and other viral death proteins, and may include:
20 virus infection assays utilizing E4/E1B Ad5 double mutants, colony formation assays using drug selection, cell killing assays using adenovirus vectors expressing E4orf4 and E4orf6, cell killing assays using retroviral vectors expressing E4orf4 and E4orf6, and the generation of cell lines expressing E4orf4 and E4orf6 under an inducible promoter (e.g., tetracycline).

E4/E1B Double Mutants

25 E4 mutations may be introduced into the mutant pm1716/2072, which is defective in production of the E1B-19K protein (McLorie, W. et al., *supra*). Such

mutants are defective for protection against p53-independent apoptosis, but the absence of 19K alone does not greatly affect viral yields. In addition, the existence of E1B-55K enhances efficient viral replication and protects against E1A-induced p53-dependent apoptosis. Thus, such E4 mutants can be grown efficiently in W162 cells, a monkey cell line which expresses the entire E4 region (Bridge, E. et al., *supra*). Viral mutants are generated by *Bam*H1 restriction endonuclease cleavage of pm1716/2072 and the series of E4 mutants, which generates DNA fragments of about 14 and 21kDa. Mixing and re-ligation of these fragments will yield recombinant viruses which are defective in both E1B-19K and E4. Such viruses can then be used in the rapid cell death assays described herein to confirm the killing efficiency of the various E4 products and to relate it to results obtained in longer term infection/killing assays. With this approach, killing of the entire cell population can be assessed rapidly.

Colony Formation Assays

A second approach is to determine the ability of the E4 death proteins to prevent cell colony formation. If the E4 proteins induce cell death effectively, colony formation should be reduced because the cells are killed. Thus, DNA constructs were made in which E4orf4 and E4orf6 were introduced into an expression plasmid (e.g., pCDNA3.1) carrying a drug selectable marker (e.g., *neo*), as described herein. Following transfection, the number of colonies formed in the presence of G418 will be counted. With this system, the E4 proteins can be assessed in the absence of other viral products and the killing process relies on inhibition of cell growth.

Adenovirus Vectors

The E4orf4 and E4orf6 proteins may be introduced into an adenovirus gene therapy vector, such as Ad5dl70-8 (Bacchetti, S. et al., 1993, *Inter. J. Oncol.* 3: 781-788). We have generated a number of such vectors using simple cloning methods. We also envision the use of adenovirus vectors in which the E4 death proteins are expressed via an inducible promoter such as tetracycline. Such vectors could be produced using standard recombinant DNA techniques. With this approach, all cells

in the population can be induced to express the E4 death proteins.

Retroviral Vectors

A preferred approach is to introduce the E4 death proteins into the mouse retroviral vector pBABE (Morgenstern and Land, 1990, Nucl. Acid Res. 18: 3587-3596) which will express high levels from the viral LTR. Such vectors also contain drug selection markers which are useful for additional studies. The E4 death protein cDNAs are cloned into pBABE and viruses prepared in cells which contain the mouse virus receptor. Virus is then harvested from the cell supernatant and used to infect mouse cells or human lines expressing the virus receptor. With this approach, the death proteins can be introduced into all cells in the culture, and expression from the vector is prolonged and would detect killing functions that require extended time periods. In addition, pBABE vectors in which the inserted cDNA is driven by an inducible promoter (e.g., tetracycline) may be constructed by standard recombinant DNA techniques. Utilization of such a recombinant virus allows the simultaneous expression of inserted DNA.

Cell Lines Expressing E4 Death Proteins

A number of human and rodent cell lines are established which express the E4 death proteins under an inducible promoter, namely tetracycline. In one example, *tet* promoters exist which are activated by addition of tetracycline. These promoters can be PCR amplified and inserted into the pCDNA vectors expressing the E4orf4 and E4orf6 proteins described herein. These plasmids can then be transfected into desired cell lines, followed by incubation of the transfected cell lines in G418 containing growth media. Cells resistant to G418 may then be cloned by limiting dilution, and individual clones induced by the addition of tetracycline. Expression level of individual clones may then be assessed utilizing antisera specific for E4orf4 and E4orf6. The advantage of this inducible expression system is that all cells can be exposed to the death proteins simultaneously following induction.

All cell killing assays described above may be carried out with E4orf4 and

E4orf6 either alone or in combination with each other or additional proteins, including other viral products, activated cellular or viral oncogenes, or suppressors of apoptosis.

IX. Selectivity of Cell Killing.

An important feature of an effective anti-cancer agent is the selectivity of cell killing. Clearly many toxic agents exist, but a key property is the ability to kill cancer cells selectively and leave normal tissues relatively unaffected. One approach is to target toxic compounds selectively to cancer cells through the use of immunological or biochemical targeting molecules. This approach has been attempted many times with limited success (Hall, S.S., 1995, Science 270:915-916). The present invention describes an approach in which an agent which is biologically active only in cancer cells (e.g., E4orf4 or E4orf6, or a combination thereof) is used to selectively induce death in cancer cells. The E4orf4 and E4orf6 proteins only kill cells which express an inappropriate growth signal, such as one provided in cancer cells by activated oncogenes or perhaps by inactivated tumor suppressors. Thus, introduction of the E4 death proteins into normal cells has little effect, whereas in cancer cells which possess signals for unregulated cell growth, these proteins are lethal.

We have shown in the present invention that the E4 death proteins (E4orf4 and E4orf6) are selective. Cells infected with a mutant Ad5 which expressed a defective E1A product lacking the ability to bind to p300/CBP and thus unable to provide a signal for unregulated DNA synthesis and cell growth, were not killed by the E4 death proteins. This result allowed the conclusion that cells must receive a signal for unregulated DNA synthesis and cell growth to be receptive to E4 killing. Two other kinds of experiments supported this conclusion. In co-transfection luciferase assays as described herein, whereas several human and monkey cell lines which exhibit 'normal' growth properties appeared to be relatively unaffected by transfection with cDNAs expressing E4orf4 and E4orf6 and exhibited high levels of luciferase activity, Ad5-transformed tumorigenic human 293 cells expressed diminished luciferase

activity when co-transfected with E4orf4 and E4orf6. As was the case in Figs. 13B and 14, the killing potential in these assays was again significantly greater with E4orf4 than with E4orf6.

Likewise, colony inhibition assays were conducted in which cells were co-
5 transfected with cDNAs expressing one of the E4 death proteins or a control, as well as a second construct encoding either the *neo* or puromycin drug selectable marker. In human 293 and Saos-2 osteosarcoma-derived human cells and in E1A-transformed mouse 1A.A6 cells, co-transfection with E4orf4 cDNA reduced colony formation by about 80% relative to a control vector lacking the E4orf4 coding sequence. The
10 reduction was less with E4orf6: about 30% in the case of Saos-2 cells. These colony inhibition approach experiments further demonstrated that transformed cells were more susceptible to E4orf4- or E4orf6-induced killing than non-transformed cells.

X. Establishment of Killing Specificity for Cancer Cells.

Using the killing assays and constructs described herein, we are able to test the
15 specificity of killing of the E4orf4 and E4orf6 death proteins and determine the degree of cell killing in normal and transformed cancer cells. In the case of rodent cells, normal cells may include primary kidney epithelial cells from normal rats or from normal or p53-null mice. A variety of transformed or tumor derived rodent cells may also be tested. In the case of adenovirus or retrovirus experiments, studies are
20 conducted on density-inhibited or serum-deprived rodent cells to establish if cell growth rate affects killing. In the case of human cells, similar studies may be conducted on a variety of "normal" human cell lines, including MRC5, IMR90, WI-38, and on a variety of transformed or tumor derived cells, especially those which have been characterized for p53 expression. The number of such lines may be
25 extended by appraising p53 expression with, for example, Western blotting analysis techniques using commercially available p53-specific antibodies (from, e.g., Santa Cruz Biotechnology, Inc.), to include a wide variety of human tumors in order to

establish the range of cancer types killed by the E4 death proteins. These various cell lines are commercially available from the ATCC (Rockville, Maryland). These studies provide clear information on the specificity of killing for neoplastic cells.

5 A particularly important variation on these experiments is to co-express, in normal cells, a variety of activated human oncogenes in concert with the E4 death proteins. Such studies directly test the degree of enhanced cell killing in the presence of growth altering oncogene products.

XI. Identification of the Minimal Killing Domains of the E4 Death Proteins.

10 To selectively kill cancer cells, it is desirable to localize the minimal killing domains of the E4orf4 and E4orf6 proteins. The minimal killing domains are likely located within the known active sites of these proteins, although there may be, as yet, unknown functions for these proteins. Peptide fragments encompassing domains with or without known function of the E4orf4 and E4orf6 proteins are generated and
15 assessed for cell killing capabilities.

The only known function of E4orf4 is to bind to and activate PP2A (Kleinberger and Shenk, 1993, J. Virol. 67: 7556-7560). Such activation may result in the dephosphorylation of components of the cell death pathway, resulting in induction of apoptosis. E4orf6 is also known to form complexes with at least two proteins, p53
20 and E1B-55K, and to carry out a number of different functions involved in the viral infectious cycle (Sarnow, P. et al., 1984, J. of Virol. 49: 692-700; Dobner, T. et al., 1996, Science 272:1470-1473).

A series of mutants are constructed to map the regions within E4orf4 and E4orf6 which are responsible for induction of p53-independent apoptosis. Deletion
25 mutants are generated which eliminate increasing stretches of the N- and C-termini of these proteins. In addition, in-frame deletion of selected internal hydrophilic regions are created and point mutants at critical conserved residues are generated. Methods for the generation of such mutants are well known in the art (see, for example,

Ausubel, F. et al., *supra*). For example, a deletion mutant eliminating the N-terminus of the E4orf4 proteins may be generated by PCR amplifying the E4orf4 cDNA containing pCDNA3.1 vector described herein with primers corresponding to the desired C-terminal portion of the protein. Preferably, the PCR primers include
5 restriction endonuclease recognition sequences at their 5' ends which facilitate insertion of the PCR product into an expression cloning vector (e.g., pCDNA3.1). The resulting plasmid may then be subjected to DNA sequence analysis. Expression may be assessed by transfection of the fragment-encoding plasmid into a eukaryotic cell line (e.g., COS cells), followed by detection of the desired fragment by Western
10 blotting analysis of transfected cell lysates with anti-E4orf4 antibodies.

Introduction of mutants into, for example, pBABE retroviral vectors or application of standard luciferase assays allow rapid determination of the killing potential of the fragments in cells, such as those described herein, which are known to be readily killed by these proteins. Once the killing regions are identified they are fine
15 mapped through the generation of more specific in-frame deletions or point mutations. Protocols for these techniques are well known in the art of molecular biology. In addition, various DNA mutation kits are commercially available, such as Clontech's Transformer™ Site-Directed Mutagenesis Kit. The identification of such killing domains also provides important information on the mechanism of killing as
20 sequences may be compared with PP2A binding, in the case of E4orf4, or one of the several known biological activities of E4orf6.

Other preferred fragments and mutants thereof are those which include conservative amino acid substitutions. Preferable fragments include fragments, or mutants thereof, which increase the biological activity (i.e., cell killing activity) or
25 stability (i.e., half-life) of the E4 death proteins.

XII. Generation of anti-E4orf4 and anti-E4orf6 Antibodies.

Once introduced into a cell, determination of expression of E4orf4 or E4orf6,

or fragments or mutants thereof is desirable. One method for the determination of expression is the utilization of antibodies specific toward these proteins. High titre antisera directed against both the – and C-termini of E4orf6 has been generated, and similar sera directed against the – and C-termini of E4orf4 are also produced. These
5 antisera are produced using both fusion products and synthetic peptides corresponding to the E4orf4 and E4orf6 death proteins as immunizing antigens according to standard protocols (see, e.g., Ausubel, F. et al., *supra*). Specificity and titre of antisera may be assessed by standard molecular biology techniques such as Western blotting analysis, immunoprecipitation, and ELISA.

10 **XIII. Identification and Generation of Peptide Mimetics Corresponding to the E4orf4 and E4orf6 Killing Domains.**

One efficient method to treat cancer patients with the E4 death proteins' functions is to generate mimetics which possess the killing effects of the E4orf4 and E4orf6 proteins. The mechanism of toxicity of neither protein appears to contain
15 enzymatic activity, and they function through complex formation with cellular proteins.

Once the killing domains have been mapped, synthetic peptides corresponding to these regions may purchased from commercially available sources (such as the Sheldon Biotechnology Centre, McGill University, Montreal, Que., Canada), and
20 tested for toxicity in the cell killing assays described herein. Such peptides may be taken up directly by cells in culture or delivered to the cells by a variety of methods, including lipid vesicles or electroporation. In addition, nucleic acid sequences encoding these peptides may be subcloned into the cloning site of an expression cloning vector (e.g., pCDNA3.1) and the plasmid DNA introduced to the cell of
25 interest by various transfection methods known in the art (e.g., electroporation, DEAE-dextran, calcium phosphate). DNA encoding peptides corresponding to the killing domains of E4orf4 and E4orf6 may also be incorporated into coding sequences

of fusion proteins and the mimetic delivered by transfection of the fusion protein encoding expression vector or fusion protein encoding viral vectors.

It will be understood that peptide mimetics of E4orf4 and E4orf6, or DNA encoding these peptides, may be used in concert. For example, two or more different E4orf4 peptides which correspond to different regions of the E4orf4 killing domain may be introduced into the same population of cells. E4orf4 peptide mimetic(s) may also be introduced with one or more peptide mimetics corresponding to the E4orf6 killing domain into the same population of cells.

Peptides, or combinations thereof, may be screened for efficacy and effective dose requirements using the various cell killing assays described herein. For example, various concentrations of peptide may be introduced with the luciferase-expressing plasmid (pRSV-luciferase) described herein into 1A.A3 cells. Forty-eight hours following introduction, cells are lysed and luciferase activity assessed using the Promega luciferase assay kit (Catalog #E1500). A cell killing peptide will induce cell killing (i.e., reduce luciferase activity) in 1A.A3 cells as compared to 1A.A3 cells transfected with a non-death inducing control (e.g., crmA).

DNA encoding potential peptide mimetics of E4 death proteins may also be identified by hybridization of the DNA to the nucleotide sequences encoding E4orf6 (SEQ ID NO.: 1) and E4orf4 (SEQ ID NO.: 3), provided on Figs. 15 and 16, respectively. In one particular example of this approach, DNAs may be identified by an ability to hybridize to the adenovirus nucleotide sequences under high stringency conditions. High stringency conditions may include hybridization at about 40°C in about 2XSSC and 1%SDS, followed by a first wash at about 65°C in about 2XSSC and 1%SDS, and a second wash at about 65°C in about 1XSSC. Other hybridization stringency conditions, both high and low, are defined in the art (see, for example, Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, 1989, or Ausubel, F. et al., *supra*).

Once identified, a DNA which hybridizes to the nucleotide sequences encoding

E4orf4 or E4orf6 may be used to generate a polypeptide product by standard techniques. For example, the DNA may be subcloned into pCDNA3.1 and the resulting plasmid transfected into, for example, COS cells (commercially available from the ATCC), to produce recombinant polypeptide). This polypeptide product may then be screened
5 for E4orf4 or E4orf6 biological activity using the various cell death assays described herein.

XIV. Production of Non-Peptide Mimetics.

The identification of minimal peptide killing sequences allows the generation of non-peptidic mimetics. Techniques for generating such non-peptidic mimetics of
10 the killing domains of E4orf4 and E4orf6 are standard chemistry techniques and well known to one skilled in the art of combinatorial chemistry. The efficacies of these non-peptide mimetics may be assayed similarly to peptide mimetics of E4orf4 and E4orf6 killing domains.

XV. Screens for Additional Reagents which Mimic E4 Death Proteins.

15 It is understood that different compounds may have the same mechanism of action without concomitant similarity in size and/or structure. Accordingly, although peptide and non-peptide mimetics based upon the killing domains of E4orf4 and E4orf6 are likely to induce an E4 death protein-like apoptosis in transformed cells, non-mimetic reagents may also have this capability. Utilizing the cell death assays
20 described herein, such reagents may be identified.

In one approach, 1A.A3 cells stably expressing a detectable protein (e.g., green fluorescent protein (GFP)) may be generated by transfecting 1A.A3 cells with pCDNA3.1 (Invitrogen) encoding GFP and then culturing the transfected cells in the presence of G418. These GFP-expressing 1A.A3 cells may then be contacted with a
25 reagent or combination thereof being tested for an ability to induce an E4 death protein cell death. Following contact, the cells may be analysed by flow cytometry for

living cells expressing GFP. A compound or reagent which induces an E4orf4 or E4orf6-like apoptosis will reduce the number of living cells expressing GFP as compared to a cell not contacted. Living cells versus non-living cells may be differentiated by size (i.e., side scatter and forward scatter profiles on a flow cytometer), or by staining with Propidium Iodide.

In another flow cytometry-based screen, reagent-contacted and non-contacted cells may be stained with Annexin V coupled to FITC (Endogen), followed by flow cytometry analysis. This type of apoptosis assay is based upon the presence of phosphatidyl-serine on the inner leaflet of the cell membrane. While a healthy cell does not bear phosphatidyl serine on its cell surface, a cell undergoing apoptosis does, as part of the apoptotic process. Annexin V binds with high affinity to phosphatidyl-serine residues, and coupling of Annexin V to FITC allows detection of apoptotic cells by flow cytometry.

It is understood that cell death analyses need not be by flow cytometry. For example, DNA fragmentation may be used as a method to detect apoptotic cells.

In a preferred embodiment, screens for reagents which induce an E4 death protein-like cell death are rapid and high through-put. For example, GFP-expressing 1A.A3 cells may be cultured in a multi-well (i.e., a 96 well microtiter) plate. Following contact with reagents being screened for an ability to induce an E4 death protein-like cell death wherein a different reagent or combination thereof is added per well of cells, the plate may then be subjected to analysis on a 96 well plate reader for the presence of GFP. A well with reduced GFP expression compared to a control untreated well indicates a compound with an ability to induce an E4 death protein-like cell death.

XVI. Apoptosis-inducing Reagents Which Activate Protein Phosphates 2A (PP2A).

Given the well characterized function of E4orf4 in binding to and activating

PP2A (Kleinberger and Shenk, 1993, J. Virol. 67: 7556-7560), reagents which activate PP2A are useful in inducing cell death in transformed cells. Reagents which activate PP2A are readily identified using commercially available reagents. In one approach, cells, for example, human Saos-2 cells, are contacted with reagents being screened for an ability to activate PP2A. Following contact, cells are lysed, and PP2A is immunoprecipitated with commercially available anti-human PP2A antibodies (from, e.g., Upstate Biotechnology, Inc.). The PP2A immunoprecipitates may then be subjected to any of a number of standard phosphates assays known in the art. An increase in the activity of PP2A as compared to PP2A isolated from untreated cells indicates a compound which activates PP2A and is able to induce E4orf4-like cell death. These compounds may be subjected to a secondary cell killing assay screen, as described herein.

XVII. Identification of Other Viral Death Proteins.

In addition to the Ad5 E4orf4 and E4orf6 death proteins, proteins from other viruses also induce apoptosis. Thus, such proteins may also prove to have efficient anti-cancer activities which may be specific or wide range. Other adenovirus proteins, or proteins from other viruses, such as the NS protein of the B-19 human parvovirus, which is known to control replication and cell killing only in rapidly dividing cells (Ozawa, K. et al., 1988, J. of Virol. 62:2884-2889), may be assayed in the cell killing assays described herein for a specific transformed cell killing ability.

XVIII. Analysis of E4 Death Proteins of Additional Human Adenoviruses.

The present invention allows the analysis of the E4orf4 and E4orf6 proteins of selected adenoviruses among the more than 40 human serotypes for toxicity by the methods described herein. Different serotypes maintain a degree of sequence homology but also possess varying and interesting differences which may result in proteins of potentially differing potency or specificity. The corresponding E4 cDNAs

from additional adenovirus serotypes are cloned by a variety of approaches well known in the art of molecular biology. These proteins are then assessed for an ability to induce cell death in transformed cell lines using the assays described herein.

XIX. Adenovirus E4 Death Proteins to Treat Cancer.

- 5 The present invention allows the use of adenovirus E4 death proteins in the treatment of cancers, especially those cancers which are p53-negative and which are difficult to eliminate by existing therapies. This invention allows the development of non-peptidic mimetics which may be used as reagents to treat cancers without the problem of specific targeting and the complication of toxicity to normal tissues.
- 10 Converting highly evolved viral functions into chemotherapeutic agents is novel, and has great potential for this and other disease treatment regimes.

XX. Administration of E4 Death Proteins and Reagents Thereof.

- An adenovirus E4orf4 or E4orf6 protein, nucleic acid, or mimetic reagent may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in
- 15 unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer E4orf4 and/or E4orf6 proteins or E4orf4 or E4orf6-mimicking reagents (e.g., peptide or non-peptide mimetics) to patients suffering from a disease (e.g., cancer) that is caused by reduced apoptosis. Administration may begin before the patient is symptomatic. Any appropriate route of
- 20 administration may be employed, for example, administration may be parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration,
- 25 formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in Remington's Pharmaceutical Sciences (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes.

Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for E4orf4 or E4orf6 death proteins and mimetics thereof include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

If desired, treatment with an E4orf4 or E4orf6 protein, gene, or mimetic reagent may be combined with more traditional therapies for the disease such as surgery, steroid therapy, or chemotherapy for autoimmune disease; antiviral therapy for AIDS; and chemotherapy for cancer.

Dosage requirements for the administration of the E4 death proteins, or mimetics thereof, may be initially determined in the cell killing assays described herein (e.g., the luciferase assay) on cultured cells. It is understood that cells of different origin may be assessed by this method for dosage requirements. For example, a human p53-null carcinoma cell line may be acquired from the ATCC and used to determine the preferred dosages to treat patients suffering from cancers of this origin. It is understood, however, that further dosage requirements for treatment of patients may be assessed *in vivo* in each individual and may vary based upon body mass and tumor size.

XXI. Therapies.

Therapies may be designed to induce E4 death protein-mediated apoptosis in transformed cells. Apoptosis-inducing E4 death protein reagents may include, without limitation, E4orf4 and E4orf6 full length proteins, polypeptide fragments, peptide and
5 non-peptide mimetics, other analogs, or combinations thereof, E4orf4 mRNA, E4orf6 mRNA, compounds which increase the stability of E4orf4 and/or E4orf6, or any compound which increases the apoptosis-inducing activity of E4orf4 and/or E4orf6.

a) Protein Therapy

To introduce E4orf4 or E4orf 6 proteins and polypeptide fragments thereof to
10 transformed cells, it is necessary to obtain large amounts of pure polypeptide from cultured cell systems which can express the polypeptide. Delivery of the polypeptide to the affected tissues (e.g., cancerous tissues) can then be accomplished using appropriate packaging or administering systems. Alternatively, small molecule analogs may be used and administered to act as E4 death protein agonists and in this
15 manner produce a desired physiological effect. Methods for finding such analogs are provided herein.

Another therapeutic approach within the invention involves administration of recombinant E4 death protein polypeptides, either directly to the site of a desired apoptosis event (for example, by injection) or systemically (for example, by any
20 conventional recombinant protein administration technique).

b) Gene Therapy

Gene therapy is another potential therapeutic approach in which copies of DNA encoding the E4 death proteins or fragments thereof are introduced into selected tissues to successfully encode for abundant polypeptide product in affected cell types
25 (e.g., cancer cells). The DNA must be delivered to those cells in a form in which it can be taken up and encode for sufficient polypeptide product to provide effective function.

Transducing retroviral vectors can be used for somatic cell gene therapy

especially because of their high efficiency of infection and stable integration and expression. The full length DNA encoding E4orf4 and/or E4orf6, or portions thereof, can be cloned into a retroviral vector and driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest (such as neurons). Other viral vectors which can be used include
5 adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpes virus such as Epstein-Barr Virus.

Gene transfer could also be achieved using non-viral means requiring infection of cancer cells *in vitro*. This would include calcium phosphate, DEAE dextran,
10 electroporation, and protoplast fusion. Liposomes may also be potentially beneficial for delivery of DNA into a cell. Although these methods are available, many of these are lower efficiency.

Transplantation of DNA encoding the E4 death proteins or polypeptide fragments thereof into the affected cells of a patient can also be useful therapy. In this
15 procedure, DNA encoding one or both of the E4 death proteins is transferred into a cultivatable cell type, either exogenously or endogenously to the patient. These cells are then injected serotologically into the targeted tissue(s).

Retroviral vectors, adenoviral vectors, adenovirus-associated viral vectors, or other viral vectors with the appropriate tropism for cells likely to be involved in
20 diseases involving insufficient apoptosis may be used as a gene transfer delivery system for a therapeutic E4 death protein DNA construct. Numerous vectors useful for this purpose are generally known (Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis and Anderson, BioTechniques 6:608-614, 1988; Tolstoshev and Anderson, Curr. Opin. Biotech. 1:55-61, 1990; Sharp, The
25 Lancet 337:1277-1278, 1991; Cornetta et al., Nucl. Acid Res. and Mol. Biol. 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; Miller et al., Biotech. 7:980-990, 1989; Le Gal La Salle et al., Science 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995). Retroviral vectors are

particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323:370, 1990; Anderson et al., U.S. Patent No. 5,399,346). Non-viral approaches may also be employed. For example, E4 death proteins may be introduced into a cell by lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413, 1987; Ono et al., Neurosci. Lett. 117:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Meth. Enz. 101:512, 1983), asialorosonucoid-polylysine conjugation (Wu et al., J. Biol. Chem. 263:14621, 1988; Wu et al., J. Biol. Chem. 264:16985, 1989); or, less preferably, micro-injection under surgical conditions (Wolff et al., Science 247:1465, 1990).

For any of the methods of application described above, the therapeutic E4 death protein-encoding DNA construct is preferably applied to the site of the desired apoptosis event (for example, by injection). However, it may also be applied to tissue in the vicinity of the desired apoptosis event or to a blood vessel supplying the cells (e.g., cancerous cells) desired to undergo apoptosis.

In the constructs described, DNA expression can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in neural cells, lymphocytes, or muscle cells may be used to direct expression of E4orf4, E4orf6, or polypeptide fragments thereof. The enhancers used could include, without limitation, those that are characterized as tissue- or cell-specific in their expression. Alternatively, regulation of expression may be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

E4 death protein gene therapy may also be accomplished by direct administration of E4orf4 or E4orf6 mRNA to a cell that is desired to undergo undesired apoptosis. The mRNA may be produced and isolated by any standard

technique, but is most readily produced by *in vitro* transcription using encoding DNA under the control of a high efficiency promoter (e.g., the T7 promoter).

Administration of mRNA to cells can be carried out by any of the methods for direct nucleic acid administration described above.

5 While the invention has been described in connection with specific
embodiments thereof, it will be understood that it is capable of further modifications
and this application is intended to cover any variations, uses, or adaptations of the
invention following, in general, the principles of the invention and including such
departures from the present disclosure as come within known or customary practice
10 within the art to which the invention pertains and as may be applied to the essential
features hereinbefore set forth, and as follows in the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION

- (i) APPLICANT: Philip E.Branton et al.
- 5 (ii) TITLE OF THE INVENTION: Adenovirus E4 Protein For
Inducing Cell Death
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
- 10 (A) ADDRESSEE: Clark & Elbing LLP
(B) STREET: 176 Federal Street
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02110
- 15 (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0
- 20 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
(B) FILING DATE: 3 July 1997
(C) CLASSIFICATION:
- 25 (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 60/021,273
(B) FILING DATE: 5 July 1996
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 60/028,740
(B) FILING DATE: 22 October 1996
- 30 (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Bieker-Brady, Kristina
(B) REGISTRATION NUMBER: 39,109
(C) REFERENCE/DOCKET NUMBER: 50013/002WO1
- 35 (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 617-428-0200
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(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

-54-

- (A) LENGTH: 885 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

10 ATGACTACGT CCGGCGTTCC ATTTGGCATG ACACTACGAC CAACACGATC TCGGTTGTCT 60
 CCGGCGCACTC CGTACAGTAG GGATCGTCTA CCTCCTTTTG AGACAGAAAC CCGCGCTACC 120
 ATACTGGAGG ATCATCCGCT GCTGCCCGAA TGTAACTT TGACAATGCA CAACGTGAGT 180
 TACGTGCGAG GTCTTCCCTG CAGTGTGGGA TTTACGCTGA TTCAGGAATG GGTGTGTTCCC 240
 TGGGATATGG TTCTAACGCG GGAGGAGCTT GTAATCCTGA GGAAGTGTAT GCACGTGTGC 300
 CTGTGTTGTG CCAACATTGA TATCATGACG AGCATGATGA TCCATGGTTA CGAGTCCTGG 360
 GCTCTCCACT GTCATTGTTT CAGTCCCGGT TCCCTGCAGT GTATAGCCGG CGGGCAGGTT 420
 TTGGCCGACT GGTTTAGGAT GGTGGTGGAT GGCGCCATGT TTAATCAGAG GTTTATATGG 480
 15 TACCGGGAGG TGGTGAATTA CAACATGCCA AAAGAGGTAA TGTTTATGTC CAGCGTGTGT 540
 ATGAGGGGTC GCCACTTAAT CTACCTGCGC TTGTGGTATG ATGGCCACGT GGGTTCTGTG 600
 GTCCCCGCCA TGAGCTTTGG ATACAGCGCC TTGCACTGTG GGATTTTGAA CAATATTGTG 660
 GTGCTGTGCT GCAGTTACTG TGCTGATTTA AGTGAGATCA GGGTGCGCTG CTGTGCCCGG 720
 AGGACAAGGC GCCTTATGCT GCGGGCGGTG CGAATCATCG CTGAGGAGAC CACTGCCATG 780
 20 TTGTATTCTT GCAGGACGGA GCGGCGCGCG CAGCAGTTTA TTCGCGCGCT GCTGCAGCAC 840
 CACCGCCCTA TCCTGATGCA CGATTATGAC TCTACCCCA TGTAG 885

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

25

- (A) LENGTH: 294 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

30 Met Thr Thr Ser Gly Val Pro Phe Gly Met Thr Leu Arg Pro Thr Arg
 1 5 10 15
 Ser Arg Leu Ser Arg Arg Thr Pro Tyr Ser Arg Asp Arg Leu Pro Pro
 20 25 30
 Phe Glu Thr Glu Thr Arg Ala Thr Ile Leu Glu Asp His Pro Leu Leu
 35 35 40 45
 Pro Glu Cys Asn Thr Leu Thr Met His Asn Val Ser Tyr Val Arg Gly
 50 55 60
 Leu Pro Cys Ser Val Gly Phe Thr Leu Ile Gln Glu Trp Val Val Pro
 65 70 75 80
 40 Trp Asp Met Val Leu Thr Arg Glu Glu Leu Val Ile Leu Arg Lys Cys
 85 90 95
 Met His Val Cys Leu Cys Cys Ala Asn Ile Asp Ile Met Thr Ser Met
 100 105 110
 Met Ile His Gly Tyr Glu Ser Trp Ala Leu His Cys His Cys Ser Ser
 45 115 120 125
 Pro Gly Ser Leu Gln Cys Ile Ala Gly Gly Gln Val Leu Ala Ser Trp
 130 135 140

-55-

Phe Arg Met Val Val Asp Gly Ala Met Phe Asn Gln Arg Phe Ile Trp
 145 150 155 160
 Tyr Arg Glu Val Val Asn Tyr Asn Met Pro Lys Glu Val Met Phe Met
 165 170 175
 5 Ser Ser Val Phe Met Arg Gly Arg His Leu Ile Tyr Leu Arg Leu Trp
 180 185 190
 Tyr Asp Gly His Val Gly Ser Val Val Pro Ala Met Ser Phe Gly Tyr
 195 200 205
 10 Ser Ala Leu His Cys Gly Ile Leu Asn Asn Ile Val Val Leu Cys Cys
 210 215 220
 Ser Tyr Cys Ala Asp Leu Ser Glu Ile Arg Val Arg Cys Cys Ala Arg
 225 230 235 240
 Arg Thr Arg Arg Leu Met Leu Arg Ala Val Arg Ile Ile Ala Glu Glu
 245 250 255
 15 Thr Thr Ala Met Leu Tyr Ser Cys Arg Thr Glu Arg Arg Arg Gln Gln
 260 265 270
 Phe Ile Arg Ala Leu Leu Gln His His Arg Pro Ile Leu Met His Asp
 275 280 285
 20 Tyr Asp Ser Thr Pro Met
 290

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 345 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

30 ATGTTCTTC CAGCTCTTCC CGCTCCTCCC GTGTGTGACT CGCAGAACGA ATGTGTAGGT 60
 TGGCTGGGTG TGGCTTATTC TCGGGTGGTG GATGTTATCA GGGCAGCGGC GCATGAAGGA 120
 GTTTACATAG AACCCGAAGC CAGGGGGCGC CTGGATGCTT TGAGAGAGTG GATATACTAC 180
 AACTACTACA CAGAGCGATC TAAGCGGCGA GACCGGAGAC GCAGATCTGT TTGTACACGC 240
 CGCACCTGGT TTTGCTTCAG GAAATATGAC TACGTCCGGC GTTCCATTG GCATGACACT 300
 ACGACCAACA CGATCTCGGT TGTCTCGGCG CACTCCGTAC AGTAG 345

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 114 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Val Leu Pro Ala Leu Pro Ala Pro Pro Val Cys Asp Ser Gln Asn
 1 5 10 15

-56-

Glu Cys Val Gly Trp Leu Gly Val Ala Tyr Ser Ala Val Val Asp Val
 20 25 30
 Ile Arg Ala Ala Ala His Glu Gly Val Tyr Ile Glu Pro Glu Ala Arg
 35 40 45
 5 Gly Arg Leu Asp Ala Leu Arg Glu Trp Ile Tyr Tyr Asn Tyr Tyr Thr
 50 55 60
 Glu Arg Ser Lys Arg Arg Asp Arg Arg Arg Arg Ser Val Cys His Ala
 65 70 75 80
 10 Arg Thr Trp Phe Cys Phe Arg Lys Tyr Asp Tyr Val Arg Arg Ser Ile
 85 90 95
 Trp His Asp Thr Thr Thr Asn Thr Ile Ser Val Val Ser Ala His Ser
 100 105 110
 Val Gln

What is claimed is:

CLAIMS

1. A method of increasing apoptosis in a cell, said method comprising administering to said cell an apoptosis inducing amount of an E4orf6 polypeptide or apoptotic fragment thereof.
- 5 2. The method of claim 1, wherein said apoptosis is p53-independent.
3. A method of increasing apoptosis in a mammal, said method comprising providing a transgene encoding an apoptotic E4orf6 polypeptide or an apoptotic fragment thereof to a cell of said mammal, said transgene being positioned for expression in said cell.
- 10 4. A method of increasing apoptosis in a cell, said method comprising administering a compound which increases E4orf6 biological activity.
5. The method of claim 1 or 4, wherein said cell is in a mammal.
6. The method of claim 5, wherein said mammal is a human.
7. The method of claim 1, 3, or 4, wherein said cell is in a mammal diagnosed
15 as having a disease involving insufficient apoptosis.
8. The method of claim 7, wherein said disease is cancer.
9. The method of claim 4, wherein said compound is E4orf6 mRNA.
10. The method of claim 4, wherein said compound increases stability of E4orf6.

11. A method of increasing apoptosis in a cell, said method comprising administering to said cell an apoptosis inducing amount of an E4orf4 polypeptide or an apoptotic fragment thereof.

12. The method of claim 11, wherein said apoptosis is p53-independent.

5 13. A method of increasing apoptosis in a mammal, said method comprising providing a transgene encoding an apoptotic E4orf4 polypeptide or an apoptotic fragment thereof to a cell of said mammal, said transgene being positioned for expression in said cell.

10 14. A method of increasing apoptosis in a cell, said method comprising administering a compound which increases E4orf4 biological activity.

15. The method of claim 11 or 14, wherein said cell is in a mammal.

16. The method of claim 15, wherein said mammal is a human.

17. The method of claim 11, 13, or 14, wherein said cell is in a mammal diagnosed as having a disease involving insufficient apoptosis.

15 18. The method of claim 17, wherein said disease is cancer.

19. The method of claim 14, wherein said compound is E4orf4 mRNA.

20. The method of claim 14, wherein said compound increases the stability of E4orf4.

21. A method of increasing apoptosis in a cell, said method comprising administering to said cell an apoptosis inducing amount of a composition comprising an E4orf6 polypeptide or an apoptotic fragment thereof and an E4orf4 polypeptide or an apoptotic fragment thereof .

5 22. The method of claim 21, wherein said apoptosis is p53-independent.

23. A method of increasing apoptosis in a mammal, said method comprising providing a first transgene encoding an E4orf6 polypeptide or an apoptotic fragment thereof and a second transgene encoding an E4orf4 polypeptide or an apoptotic fragment thereof to a cell of said mammal, said first transgene and said second
10 transgene being positioned for expression in said cell.

24. A method of increasing apoptosis in a cell, said method comprising administering a composition comprising a first compound which increases E4orf6 biological activity and a second compound which increases E4orf4 biological activity.

25. The method of claim 21 or 24, wherein said cell is in a mammal.

15 26. The method of claim 25, wherein said mammal is a human.

27. The method of claim 21, 23, or 24, wherein said cell is in a mammal diagnosed as having cancer.

28. The method of claim 23, wherein said first transgene and said second transgene encodes E4orf4 are linked to a constitutive promoter.

29. The method of claim 24, wherein said first compound is E4orf6 mRNA and said second compound is E4orf4 mRNA

30. The method of claim 24, wherein said first compound and said second compound increase stability of E4orf6 and E4orf4.

5 31. A pharmaceutical composition comprising substantially pure nucleic acid encoding an E4orf6 polypeptide and a pharmaceutically acceptable carrier.

32. The composition of claim 31, wherein said nucleic acid encodes E4orf6 having a conservative amino acid substitution relative to the E4orf6 sequence of Fig. 15 (SEQ ID NO.: 2).

10 33. A pharmaceutical composition comprising substantially pure nucleic acid encoding an apoptotic fragment of E4orf6.

34. The composition of claim 31 or 33, wherein said nucleic acid is in a viral vector.

15 35. A pharmaceutical composition comprising nucleic acid having the sequence of Fig. 15 (SEQ ID NO.: 1), or degenerate variants thereof, and encoding the amino acid sequence of Fig. 15 (SEQ ID NO.: 2).

36. A pharmaceutical composition comprising nucleic acid having about 50% or greater nucleotide sequence identity to the DNA sequence of Fig. 15 (SEQ ID NO.: 1), said nucleic acid encoding a polypeptide with E4orf6 biological activity.

37. The composition of claim 36, wherein said nucleotide sequence identity is 75% or greater to the DNA sequence of Fig. 15 (SEQ ID NO.: 1).

38. A pharmaceutical composition comprising a DNA sequence substantially identical to the DNA sequence shown in Fig. 15 (SEQ ID NO.: 1).

5 39. The composition of claim 31 or 33, wherein said nucleic acid is operably linked to regulatory sequences for expression of said polypeptide and wherein said regulatory sequences comprise a promoter.

40. The composition of claim 39, wherein said promoter is a constitutive promoter, is inducible by one or more external agents, or is cell-type specific.

10 41. A pharmaceutical composition comprising substantially pure mammalian E4orf6 polypeptide, or an apoptotic fragment thereof.

42. The composition of claim 41, wherein said polypeptide comprises an amino acid sequence substantially identical to an amino acid sequence shown in Fig. 15 (SEQ ID NO.: 2).

15 43. The composition of claim 41, wherein said polypeptide has a conservative amino acid substitution relative to the E4orf6 sequence of Fig. 15 (SEQ ID NO.: 2).

44. A pharmaceutical composition comprising a substantially pure polypeptide fragment of E4orf6.

20 45. A pharmaceutical composition comprising substantially pure nucleic acid encoding an E4orf4 polypeptide and a pharmaceutically acceptable carrier.

46. The composition of claim 45, wherein said nucleic acid encodes E4orf4 having a conservative amino acid substitution relative to the E4orf4 sequence of Fig. 16 (SEQ ID NO.: 4).

47. A pharmaceutical composition comprising substantially pure nucleic acid
5 encoding an apoptotic fragment of E4orf4.

48. The composition of claim 45 or 47, wherein said nucleic acid is in a viral vector.

49. A pharmaceutical composition comprising nucleic acid having the sequence of Fig. 16 (SEQ ID NO.: 3), or degenerate variants thereof, and encoding
10 the amino acid sequence of Fig. 16 (SEQ ID NO.: 4).

50. A pharmaceutical composition comprising nucleic acid having about 50% or greater nucleotide sequence identity to the DNA sequence of Fig. 16 (SEQ ID NO.: 3), said nucleic acid encoding a polypeptide with E4orf4 biological activity.

51. The composition of claim 50, wherein said nucleotide sequence identity is
15 75% or greater to the DNA sequence of Fig. 16 (SEQ ID NO.: 3).

52. A pharmaceutical composition comprising a DNA sequence substantially identical to the DNA sequence shown in Fig. 16 (SEQ ID NO.: 3).

53. The composition of claim 45 or 47, wherein said nucleic acid is operably linked to regulatory sequences for expression of said polypeptide and wherein said
20 regulatory sequences comprise a promoter.

54. The composition of claim 53, wherein said promoter is a constitutive promoter, is inducible by one or more external agents, or is cell-type specific.

55. A pharmaceutical composition comprising substantially pure mammalian E4orf4 polypeptide, or an apoptotic fragment thereof.

5 56. The composition of claim 55, said polypeptide comprising an amino acid sequence substantially identical to an amino acid sequence shown in Fig. 16 (SEQ ID NO.: 4).

57. The composition of claim 55, wherein said polypeptide has a conservative amino acid substitution relative to the E4orf4 sequence of Fig. 16 (SEQ ID NO.: 4).

10 58. A pharmaceutical composition comprising a substantially pure polypeptide fragment of E4orf6.

59. A method for identifying a compound as an E4orf6 analog, said method comprising the steps of:

- 15 a) providing a cell expressing the adenovirus E1A-289R protein, said cell not expressing any E4 proteins;
- b) contacting said cell with a candidate compound; and
- c) determining viability of said cell, wherein apoptotic death of said cell indicates a compound that is an E4orf6 analog.

20 60. A method for identifying a compound as an E4orf4 analog, said method comprising the steps of:

- a) providing a cell expressing the adenovirus E1A-289R protein, said cell not expressing any E4 proteins;

b) contacting said cell with a candidate compound; and
c) determining viability of said cell, wherein apoptotic death of said cell indicates a compound that is an E4orf4 analog.

61. The method of claim 59 or 60, wherein said cell is selected from the group
5 consisting of: 1A.A3, 1A.A6, and 1A.A12 cells.

62. The method of claim 59 or 60, wherein said viability is measured with Trypan Blue™.

63. The method of claim 59 or 60, wherein said viability is measured with a DNA fragmentation assay.
10

64. The method of claim 59 or 60, wherein said viability is measured with Annexin V binding.

65. The method of claim 59 or 60, wherein said viability is measured with Propidium Iodide
15

66. The method of claim 59 or 60, wherein said viability is measured utilizing a combination of two or more of the following: Trypan Blue™, DNA fragmentation, Annexin V binding, and Propidium Iodide.

67. The method of claim 59 or 60, wherein said cell is a cell is infected with a
20 mutant adenovirus, said mutant adenovirus incapable of expressing any E4 proteins.

68. A method for identifying a compound as an E4orf4 analog, said method comprising the steps of:

- a) providing a cell expressing protein phosphatase 2A;
- b) contacting said cell with a candidate compound; and
- c) measuring activity of said protein phosphatase 2A in said cell, an increase in said activity relative to a cell not contacted with said candidate compound indicating a compound that is an E4orf4 analog.

69. A pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which comprises E4orf6, an analog or a biologically active fragment thereof.

70. A pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutical amount of E4orf6, an analog or a biologically active fragment thereof in association with a pharmaceutical carrier.

71. A pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of a compound which induces apoptosis or other cytotoxic effects analogous to E4orf6 biological activity in association with a pharmaceutical carrier.

72. A pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which comprises E4orf4, an analog or a biologically active fragment thereof.

73. A pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of E4orf4, an analog or a biologically active fragment thereof in association with a pharmaceutical carrier.

74. A pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of a compound which induces protein phosphatase 2a in association with a pharmaceutical carrier.

5 75. The composition of claim 74, wherein said compound is an agonist of E4orf4.

76. The composition of claim 74, wherein said compound mimics E4orf4 activity.

10 77. A pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of a compound which induces apoptosis or other cytotoxic effects analogous to E4orf4 biological activity in association with a pharmaceutical carrier.

15 78. A pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which comprises E4orf6, an analog or a biologically active fragment thereof; and E4orf4, an analog or a biologically active fragment thereof.

20 79. A pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutical amount of E4orf6, an analog or a biologically active fragment thereof; and E4orf4, an analog or a biologically active fragment thereof in association with a pharmaceutical carrier.

80. A pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of a

compound which induces apoptosis or other cytotoxic effects analogous to biological activities of the E4 death proteins in association with a pharmaceutical carrier.

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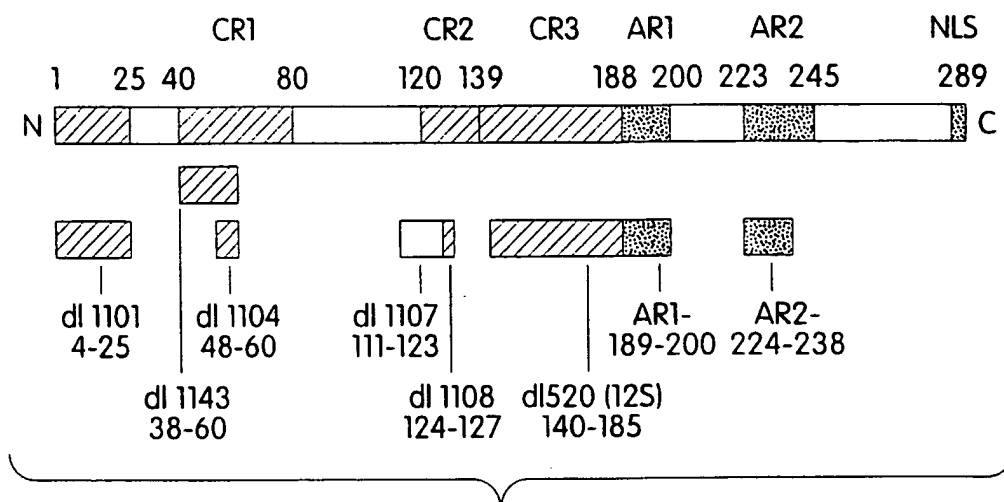


Fig. 1A

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VIRUS MUTANT	DESCRIPTION
wt Ad5	wt E1A (12S & 13S mRNAs), wt E1B. In some cases <i>dl</i> 309 which has a partial deletion of E3 was used
12S / E1B-	12S E1A mRNA only, no E1B expression
13S / E1B-	13S E1A mRNA only, no E1B expression
E1B / 55K-	wtE1A (12S & 13S mRNAs), no E1B 55K expression, wt E1B 19K
E1B / 19K-	wtE1A (12S & 13S mRNAs), no E1B 19K expression, wt E1B 55K
<i>dl</i> 1101 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
<i>dl</i> 1104 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
<i>dl</i> 1107 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
<i>dl</i> 1108 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
<i>dl</i> 1143 / 08/ E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR1- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR2- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR1- / AR2- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AD147VL / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
AD171CS / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
AD185SG / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
<i>dl</i> 1019	wt E1A, E1B, E2 and E3, no E4 expression, in Ad2
AdLacZ	no E1A or E1B expression, wt E2, E3 and E4
Ad5 <i>dl</i> 70-8	no E1A, E1B or E3 expression, wt E2 and E4
AdRSVβ-gal.11	no E1A, E1B or E4 expression, wt E2 and E3

Fig. 1B

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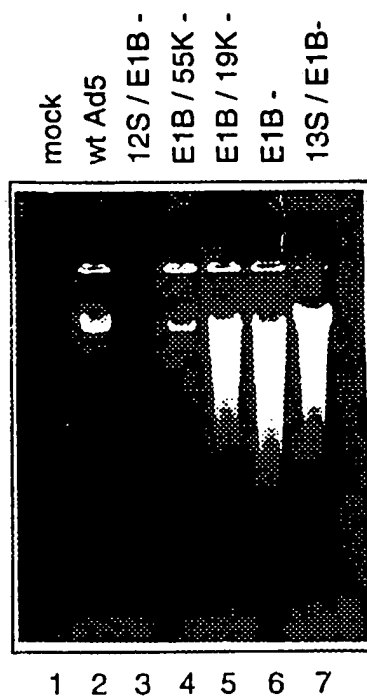


Fig. 2

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Saos-2 / Neo (2a2) cells

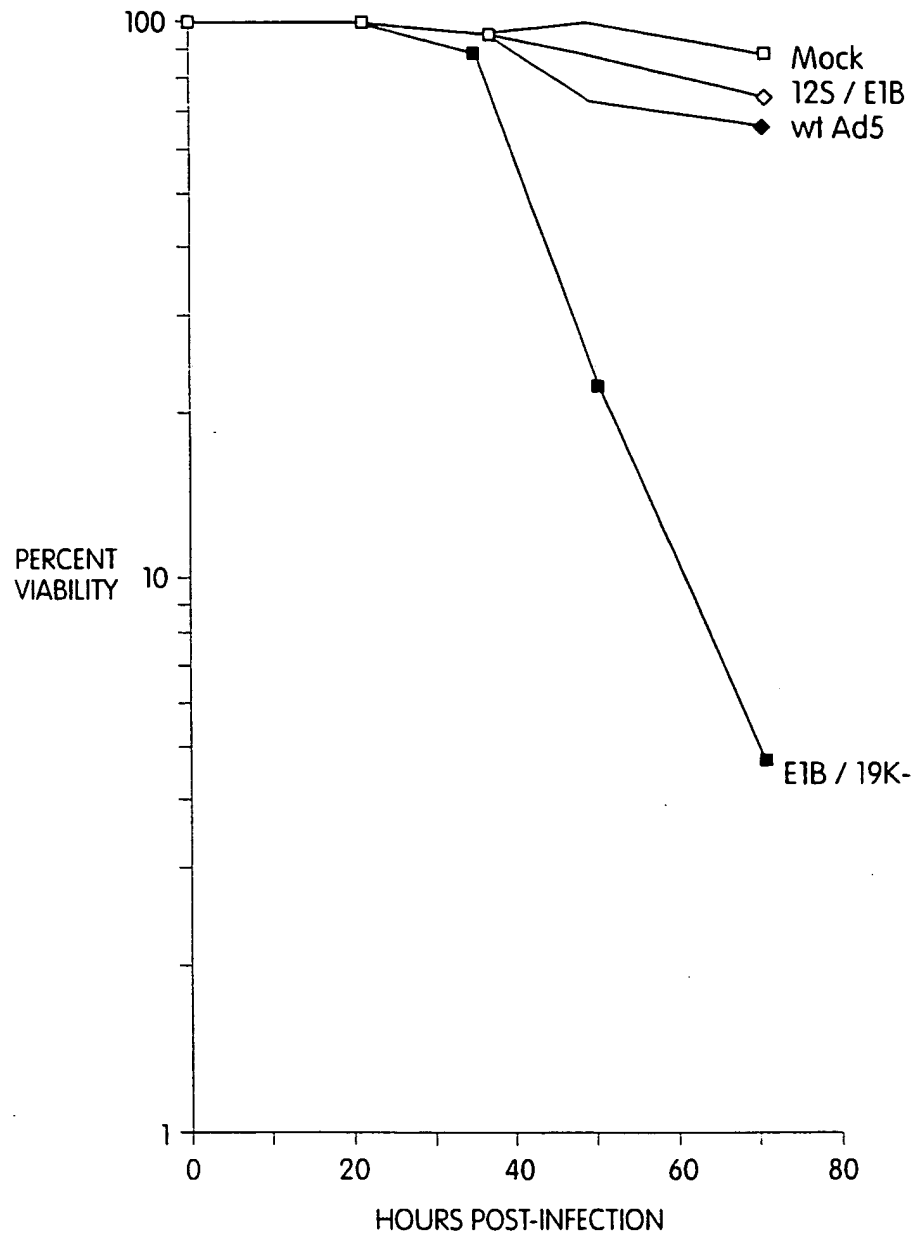


Fig. 3A

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Saos-2 / Bcl-2 (3g4) cells

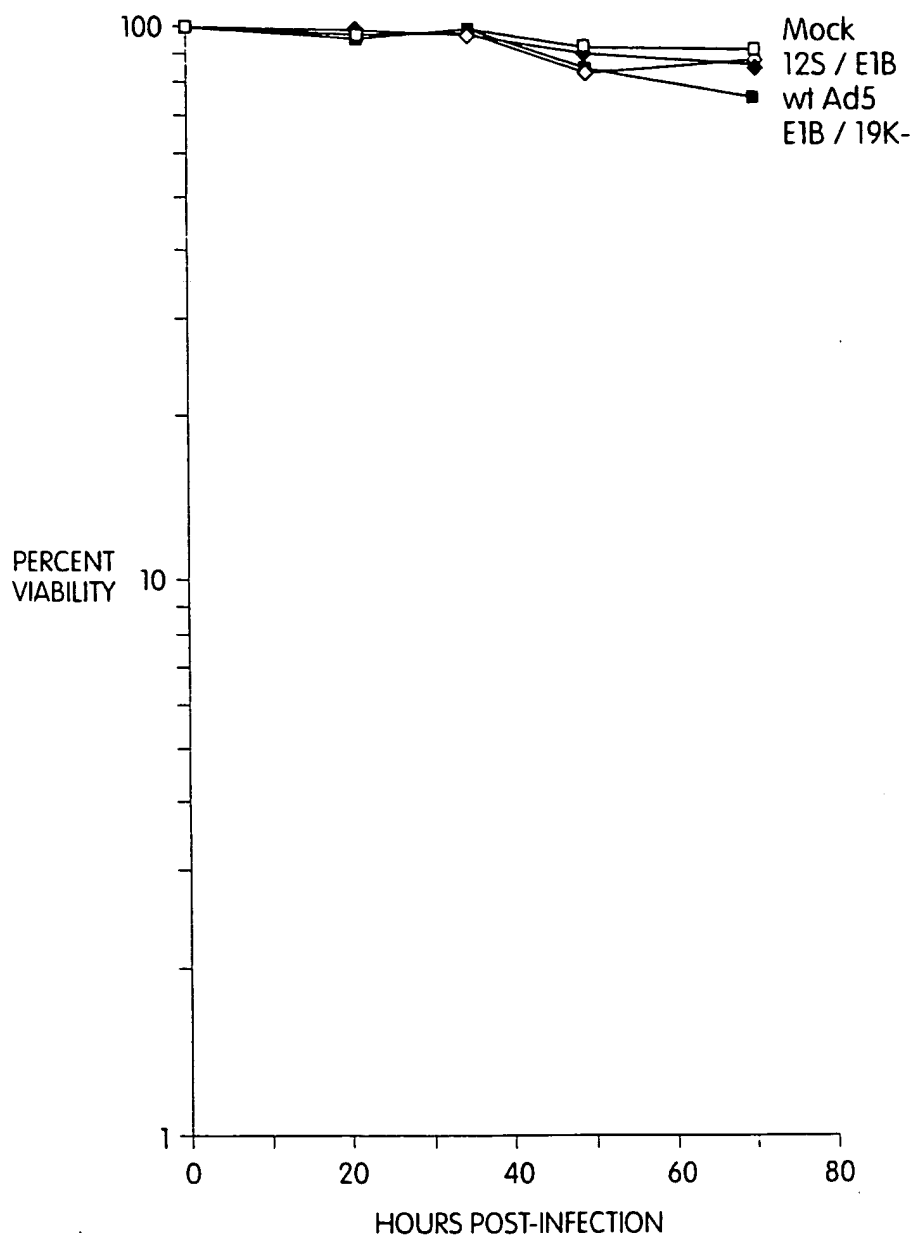


Fig. 3B

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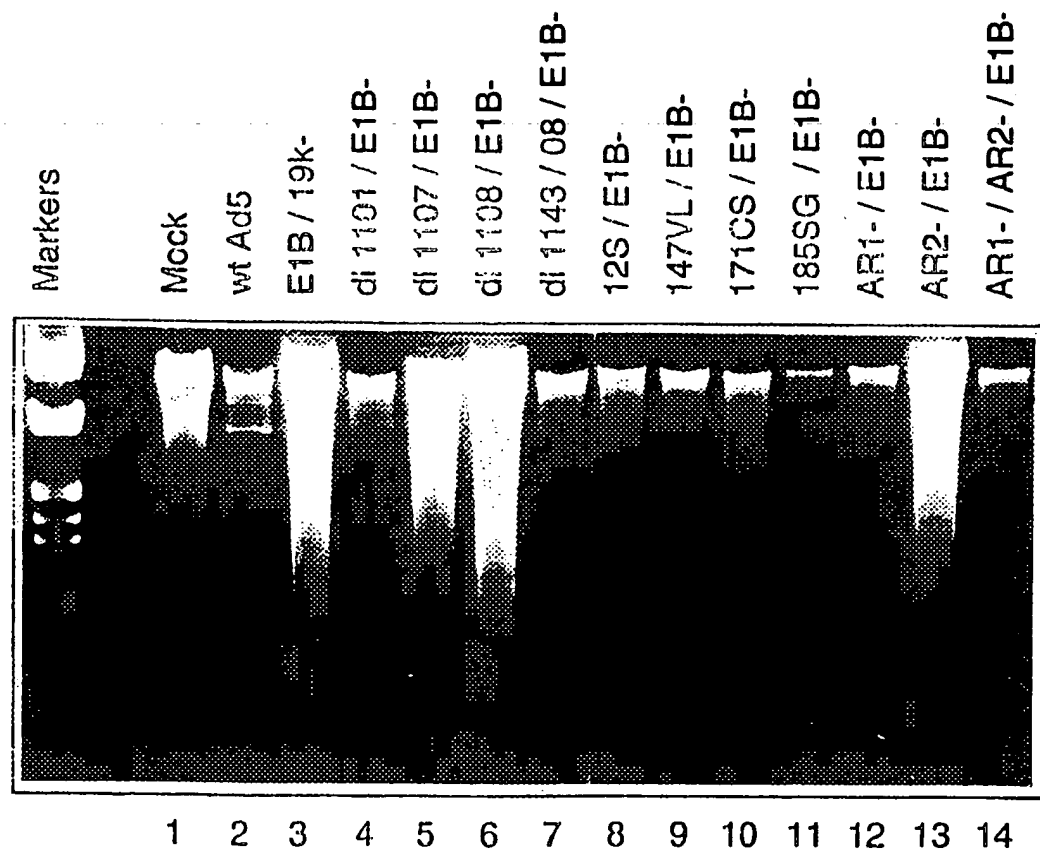


Fig. 4

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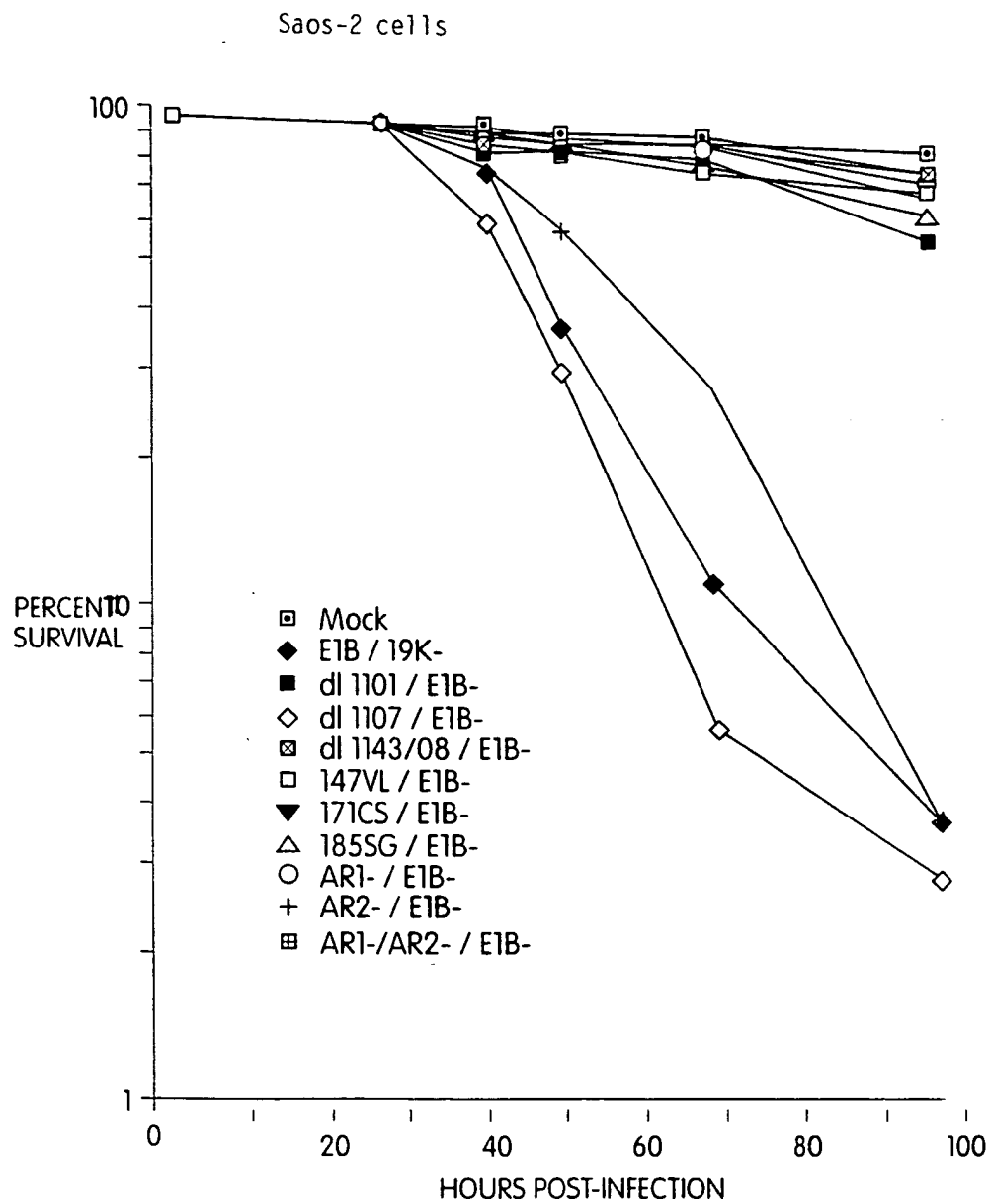


Fig. 5

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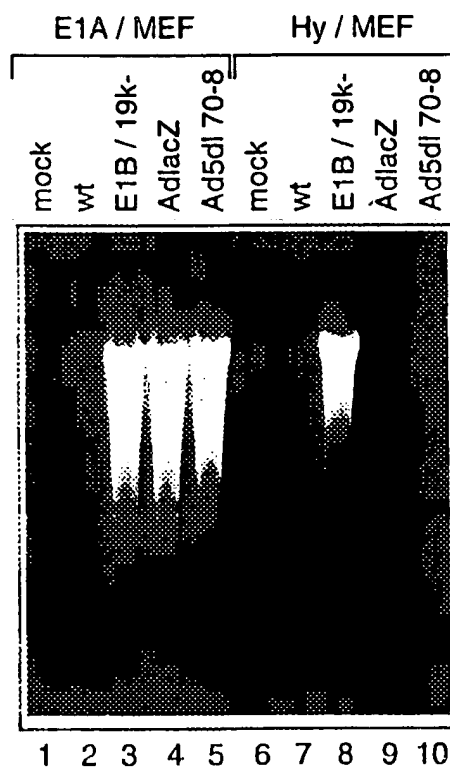


Fig. 6

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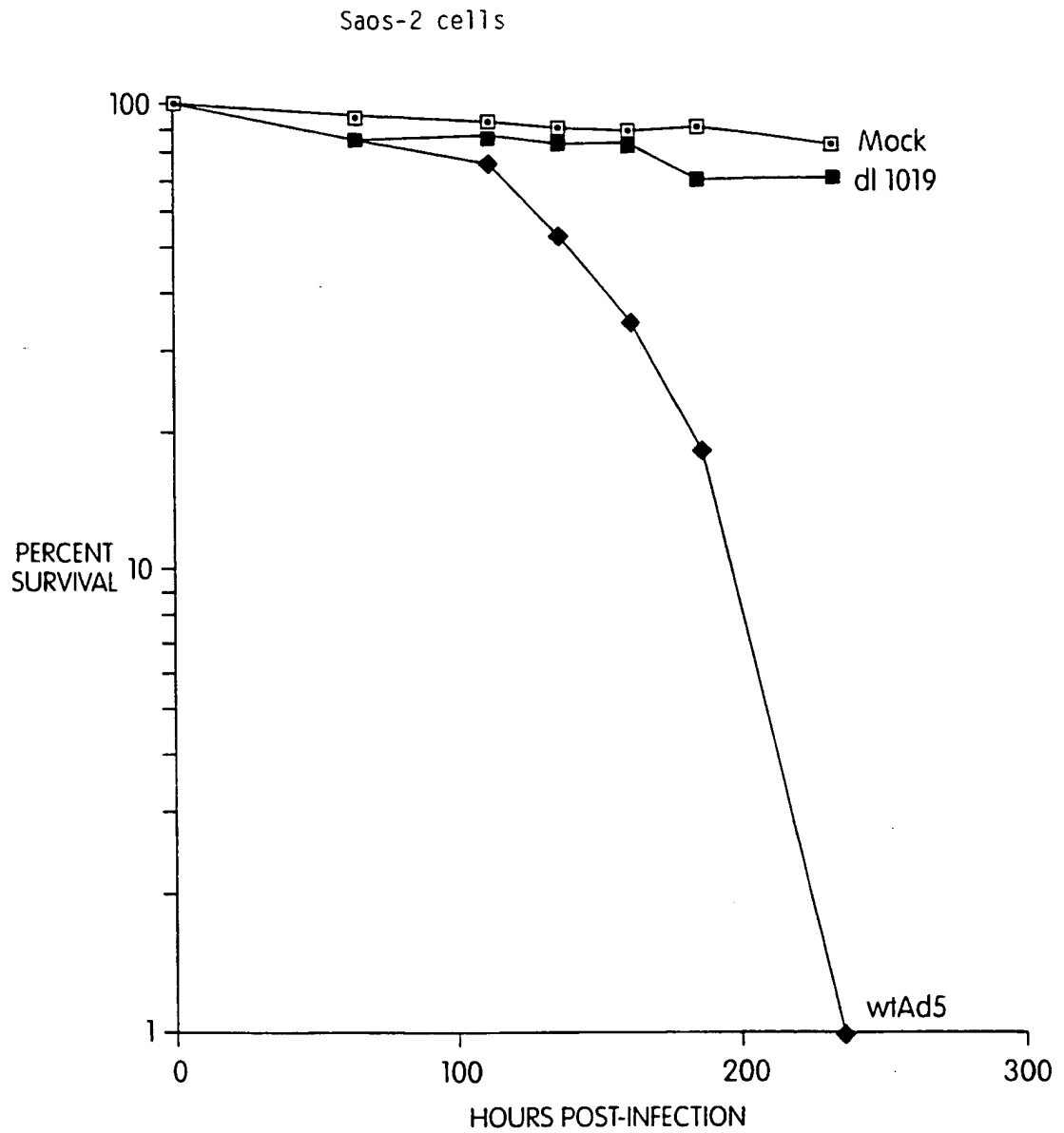


Fig. 7

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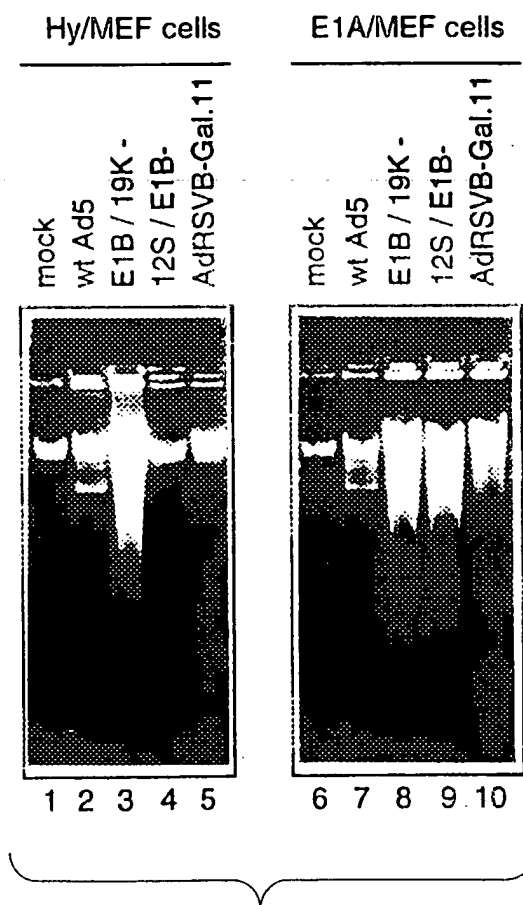


Fig. 8

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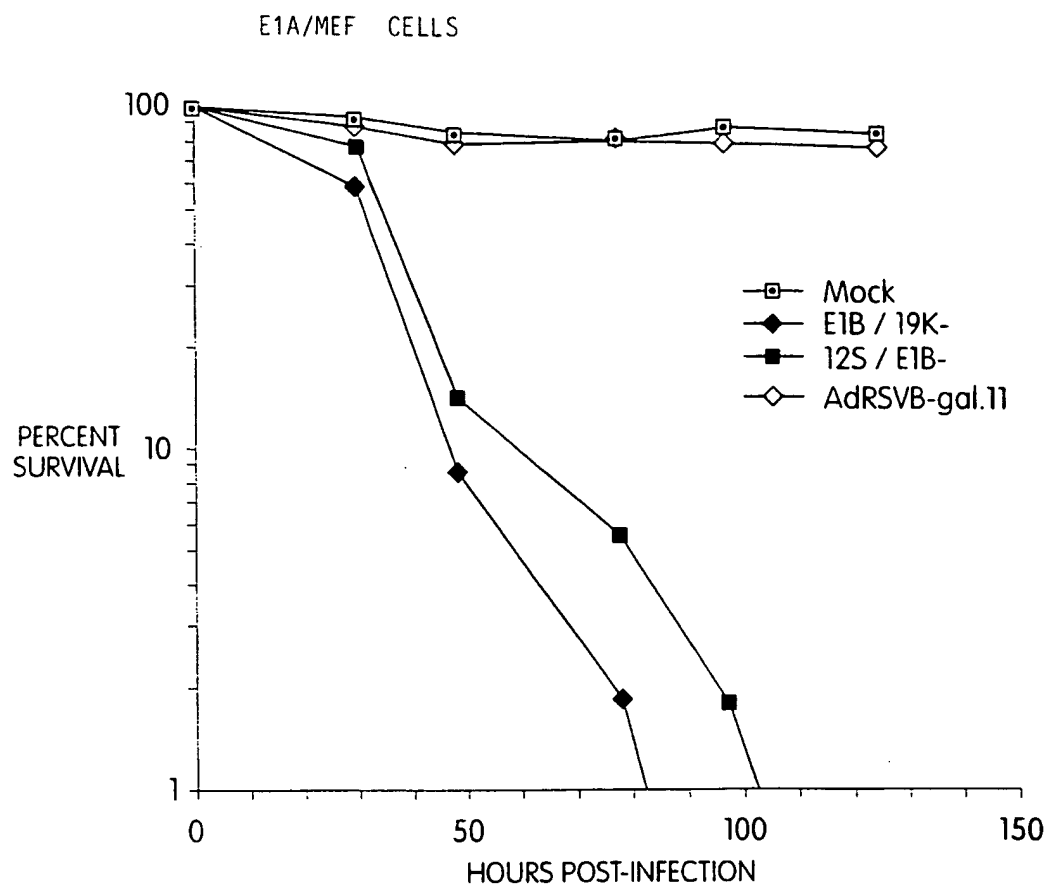


Fig. 9A

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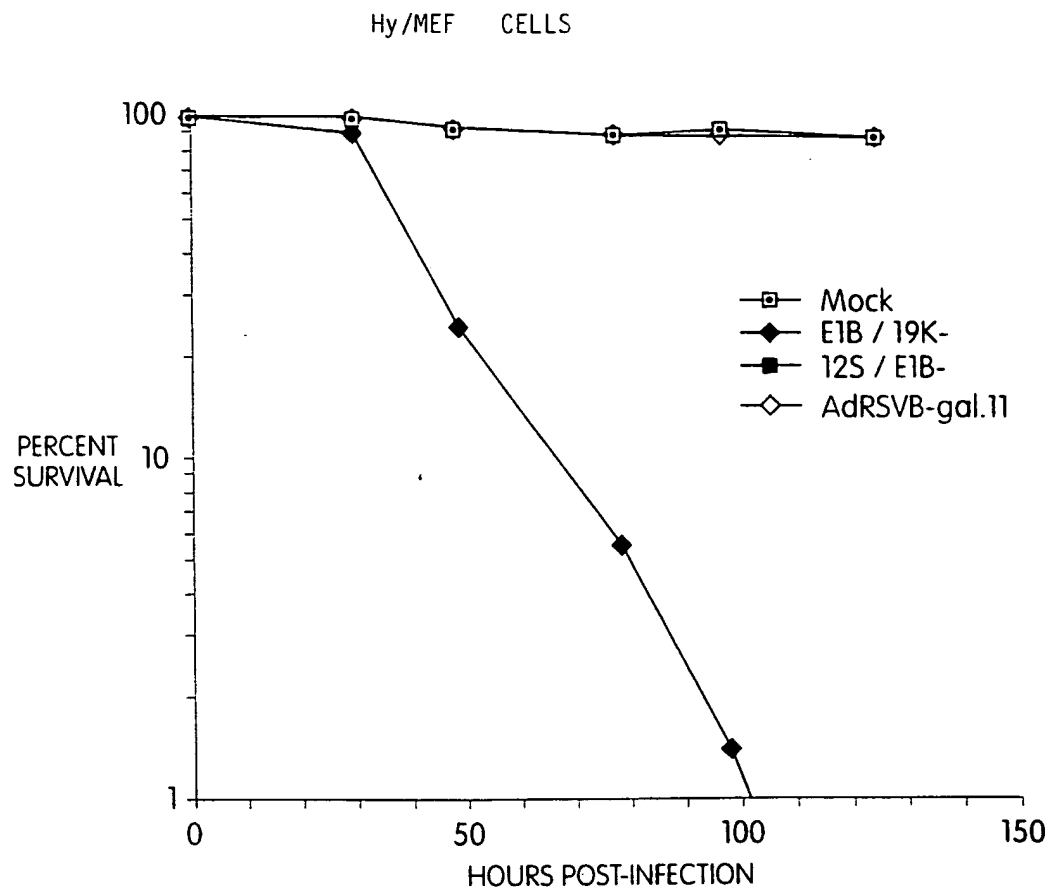


Fig. 9B

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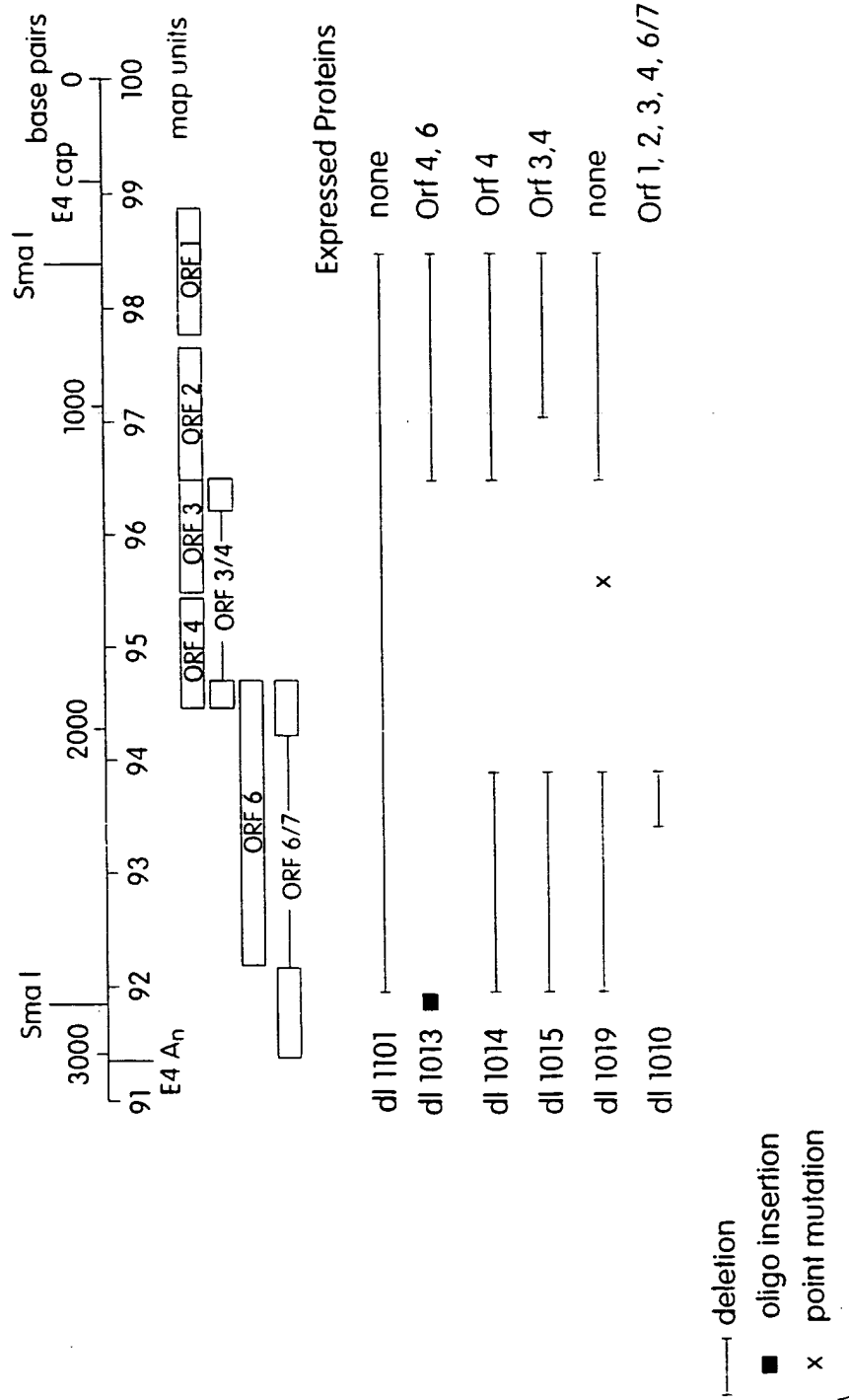


Fig. 10

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Survival Assay in Saos-2 cells

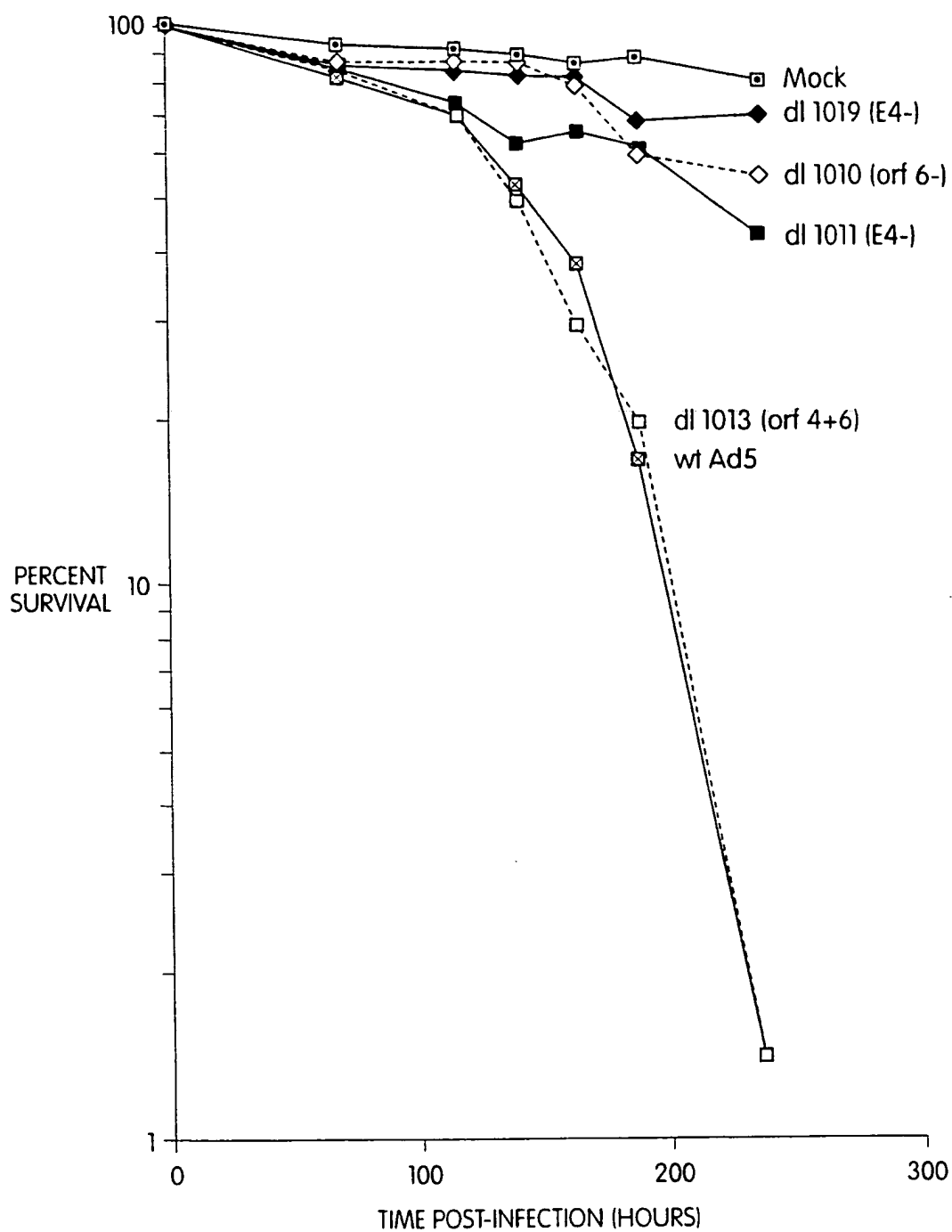


Fig. 11

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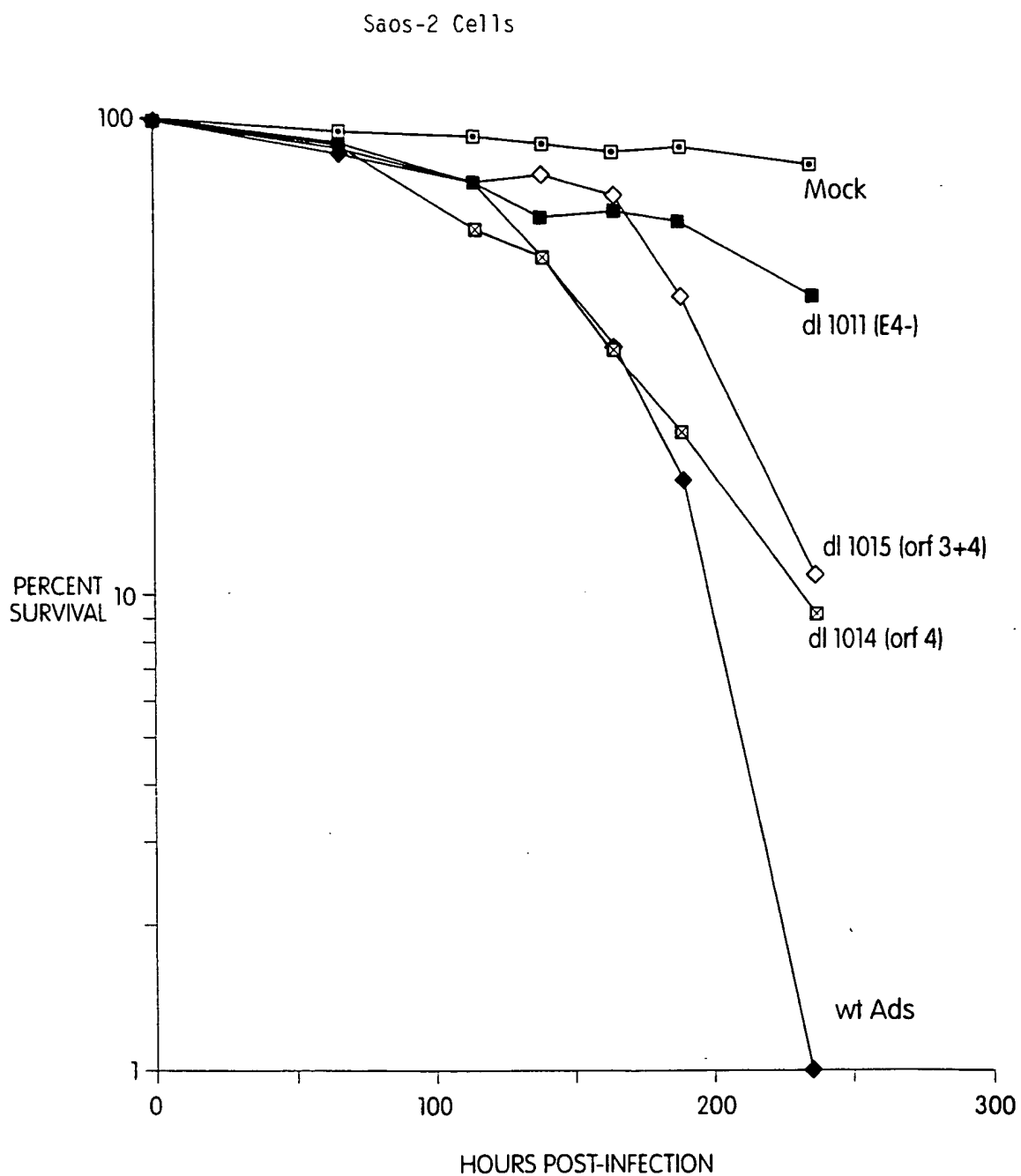


Fig. 12

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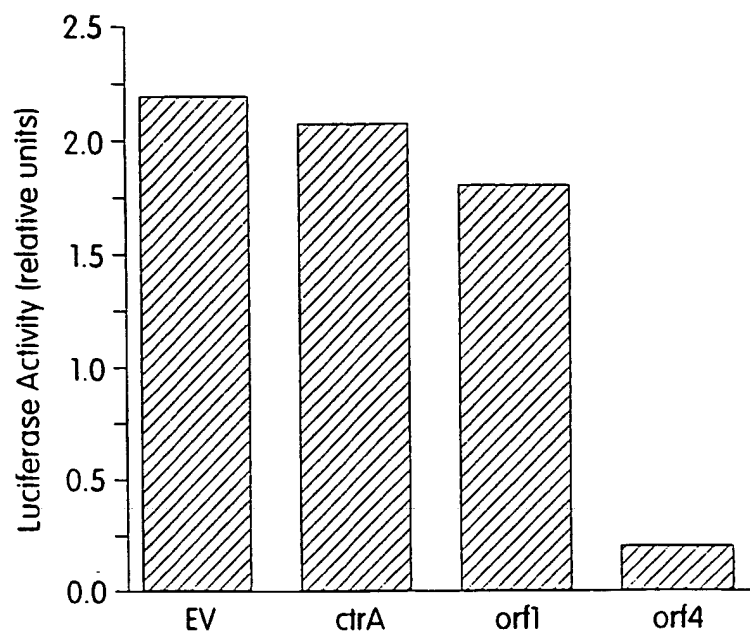


Fig. 13A

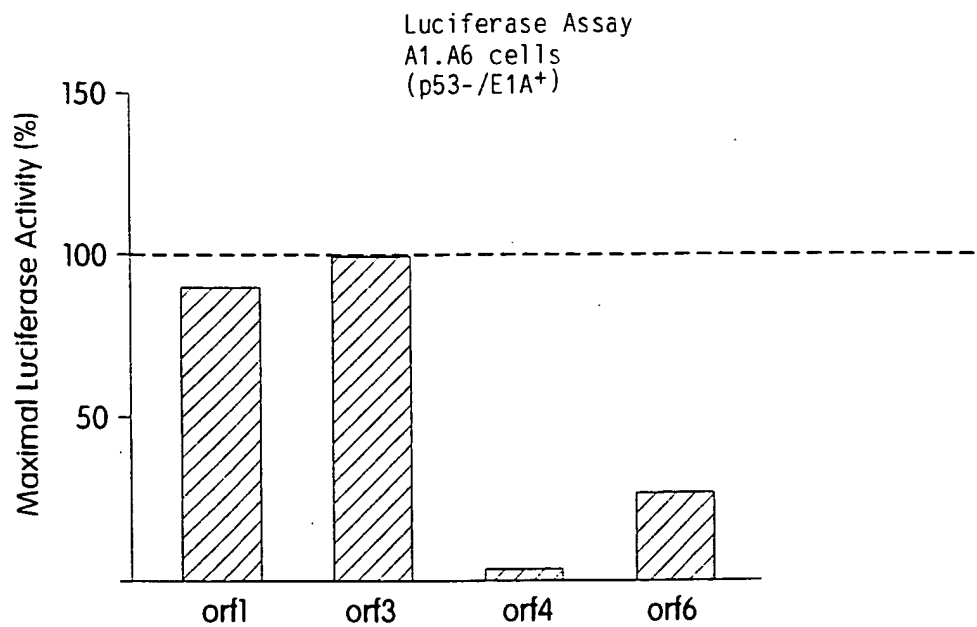


Fig. 13B

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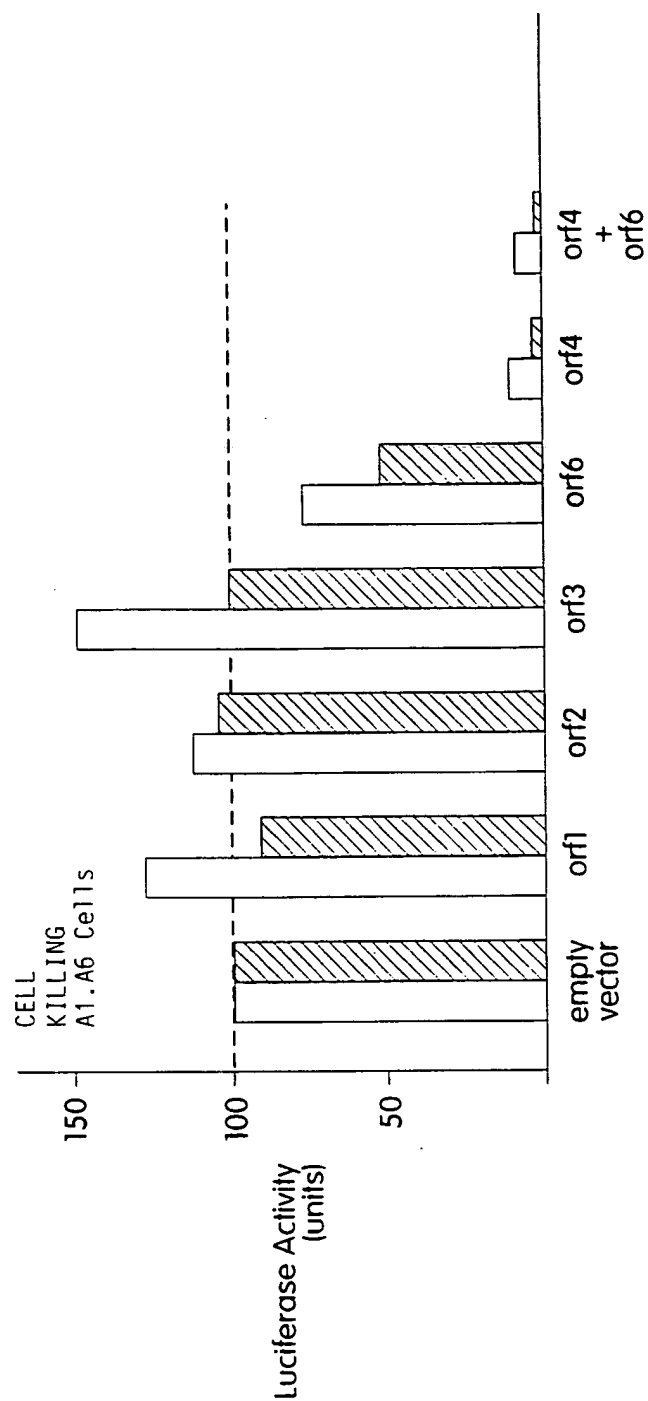


Fig. 14

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Ad5 E4orf6

10 20 30 40
atgactacgtccggcggttccatttggcatgacactacgac 40
caacacgatctcgggtgtctcggcgccactccgtacagtag 80
ggatcgtctacctccttttgagacagaaaccgcgctacc 120
atactggaggatcatccgctgctgcccgaatgtaacactt 160
tgacaatgcacaacgtgagttacgtgcgagggtcttcctg 200

210 220 230 240
cagtgtgggatttacgctgattcaggaatgggttgttccc 240
tgggatatggttctaacgcgggaggagcttgtaatcctga 280
ggaagtgtatgcacgtgtgcctgtgttgcccaacattga 320
tatcatgacgagcatgatgatccatggttacgagtcctgg 360
gctctccactgtcattgttccagtcgccggttcctgagcgt 400

410 420 430 440
gtatagccggcgggcaggttttggccagctgggttaggat 440
ggtggtggatggcgccatgtttaatcagagggttatatgg 480
taccgggagggtggtgaattacaacatgccaaaagaggtaa 520
tgtttatgtccagcgtgtttatgaggggtcgccacttaat 560
ctacctgcgcttggtgatgatggccacgtgggttctgtg 600

610 620 630 640
gtccccgccatgagctttggatacagcgccttgactgtg 640
ggattttgaacaatatgtggtgctgtgctgcagtactg 680
tgctgatttaagtgaatcaggggtgcgctgctgtgcccg 720
aggacaaggcgccttatgtgctgccccgggtgcgaatcatcg 760
ctgaggagaccactgccatgttgattcctgcaggacgga 800

810 820 830 840
gcggcgccggcagcagtttatcgcgcgctgctgcagcac 840
caccgccctatcctgatgcacgattatgactctaccccca 880
ttag 885

10 20 30 40
MTTSGVPFGMTLRPTRSRLSRRTPYSRDRLPPFETETRAT 40
ILEDHPLLPECNTLTMHNVSYVRGLPCSVGFTLIQEWVVP 80
WDMVLTREELVILRKCMHVCLCCANIDIMTSMMIHGYESW 120
ALHCHCSSPGSLQCIAGGQVLASWFRMVVDGAMFNQRFIW 160
YREVVNYNMPKEVMFMSSYFMRGRHLIYLRLWYDGHVGSV 200

210 220 230 240
VPAMSGYSAHCGILNNIVLCCSYCADLSEIRVRCCAR 240
RTRRLMLRAVRRIAEETTAMLYSCRTERRRQOFIRALLQH 280
HRPILMHDYDSTPM. 295

Fig.15

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10 20 30 40
 MVLPALPAPPVCDSDNECVGWLGVAYSADVVDVIRAAAHG 40
 VYIEPEARGLDALREWIYYNYTTERSKRRDRRRRSVCHA 80
 RTWFCFRKYDYVRRSIWHDTTTNTISVVSASHVQ. 115

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71)(72) Applicants and Inventors: BRANTON, Philip, E. [CA/CA]; 266 Macaulay, St. Lambert, Quebec J4R 2G9 (CA). SHORE, Gordon, C. [CA/CA]; 4842 Wilson, Montreal, Quebec H3X 3P2 (CA). TEODORO, Jose, G. [CA/CA]; 3484 Stanley #610, Montreal, Quebec H3A 1S1 (CA). MARCELLUS, Richard, C. [CA/CA]; 3504 Durocher #2, Montreal, Quebec H2X 2E5 (CA). LAVOIE, Josee, N. [CA/CA]; 1100 Dr. Penfield #1416, Montreal, Quebec H3A 1A8 (CA).		(88) Date of publication of the international search report: 12 March 1998 (12.03.98)	
(54) Title: ADENOVIRUS E4 PROTEINS FOR INDUCING CELL DEATH			
(57) Abstract			
<p>The present invention relates to a pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which includes adenovirus E4orf4, an analog or a biologically active fragment thereof and/or adenovirus E4orf6, an analog or a biologically active fragment thereof. There is also provided a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of adenovirus E4orf4, an analog or a biologically active fragment thereof and/or adenovirus E4orf6, an analog or a biologically active fragment thereof in association with a pharmaceutical carrier. Included in the invention are methods for identifying compounds as analogs of the adenovirus E4 death proteins.</p>			

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INTERNATIONAL SEARCH REPORT

Interr. Application No

PC1/IB 97/01041

A. CLASSIFICATION OF SUBJECT MATTER

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 96 14061 A (CELL GENESYS INC) 17 May 1996	31-54
Y	see page 13, line 13 - line 19; claims 1-59	1,4,9, 11,14, 21,24, 29, 55-58, 68-80
X	WO 96 13596 A (RHONE POULENC RORER SA ;YEH PATRICE (FR); PERRICAUDET MICHEL (FR);) see claims 1-37	31-54
X	BRIDGE E ET AL: "Adenovirus early region 4 and viral DNA synthesis." VIROLOGY, APR 1993, 193 (2) P794-801, UNITED STATES, XP002051623 see the whole document	31-54
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Date of mailing of the international search report

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KETNER G ET AL: "Complementation of adenovirus E4 mutants by transient expression of E4 cDNA and deletion plasmids." NUCLEIC ACIDS RES, APR 25 1989, 17 (8) P3037-48, ENGLAND, XP002051624 see the whole document ---	31-54
Y	TEODORO JG ET AL: "Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53-independent mechanisms." ONCOGENE, AUG 3 1995, 11 (3) P467-74, ENGLAND, XP002051625 see page 472, left-hand column, paragraph 2 - line END ---	1,4,9, 11,14, 21,24, 29, 69-73, 77-80
Y	HERISSE J ET AL: "Nucleotide sequence of adenovirus 2 DNA fragment encoding for the carboxylic region of the fiber protein and the entire E4 region." NUCLEIC ACIDS RES, AUG 25 1981, 9 (16) P4023-42, ENGLAND, XP002051626 see figure 2 ---	55-58
Y	KLEINBERGER T ET AL: "Adenovirus E4orf4 protein binds to protein phosphatase 2A, and the complex down regulates E1A-enhanced junB transcription." J VIROL, DEC 1993, 67 (12) P7556-60, UNITED STATES, XP002051627 cited in the application see the whole document ---	68,73-76
P,X	MARCELLUS, RICHARD C. ET AL: "Adenovirus type 5 early region 4 is responsible for E1A-induced p53-independent apoptosis" J. VIROL. (1996), 70(9), 6207-6215 CODEN: JOVIAM;ISSN: 0022-538X, XP002051628 see the whole document ---	1,2,11, 12,14, 21,22, 24,31-54
P,X	WO 96 22378 A (RHONE POULENC RORER SA ;DEDIEU JEAN FRANCOIS (FR); LATTA MARTINE) () see abstract; claims 1-50 ---	31-34, 39,40
P,X	WO 96 39530 A (UNIV PENNSYLVANIA ;WILSON JAMES M (US); FISHER KRISHNA J (US); GAO) see abstract; claims 1-23 -----	31-34, 39,40

INTERNATIONAL SEARCH REPORT

In international application No.
PCT/IB 97/01041

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 3 5-8 13 15-18 23 25-28 and 1 2 4 9-12 14 19-22 24 29 30 ,as far as they concern an in vivo method , are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter Application No

PCT/IB 97/01041

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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